

## PATENT COOPERATION TREATY

PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents  
United States Patent and Trademark  
Office  
Box PCT  
Washington, D.C.20231  
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

<b>Date of mailing</b> (day/month/year) 05 May 2000 (05.05.00)	
<b>International application No.</b> PCT/US99/21591	<b>Applicant's or agent's file reference</b> 27779/35932/PCT
<b>International filing date</b> (day/month/year) 16 September 1999 (16.09.99)	<b>Priority date</b> (day/month/year) 17 September 1998 (17.09.98)
<b>Applicant</b> HARRIS, Jeffrey et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

13 April 2000 (13.04.00)

☐ in a notice effecting later election filed with the International Bureau on:
2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

<p>The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland</p> <p>Facsimile No.: (41-22) 740.14.35</p>	<p>Authorized officer</p> <p>Christelle Croci</p> <p>Telephone No.: (41-22) 338.83.38.-</p>
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# PATENT COOPERATION TREATY

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

## PCT

### NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT (PCT Rule 71.1)

To:

Uhl, Jill E.  
MARSHALL, O'TOOLE, GERSTEIN,  
MURRAY & BORUN  
6300 Sears Tower  
233 South Wacker Drive  
Chicago, Illinois 60606-6402  
ETATS-UNIS D'AMERIQUE

Date of mailing  
(day/month/year) 15.01.2001

Applicant's or agent's file reference  
27779/35932/PCT

#### IMPORTANT NOTIFICATION

International application No.  
PCT/US99/21591

International filing date (day/month/year)  
16/09/1999

Priority date (day/month/year)  
17/09/1998

Applicant  
ZONAGEN, INC. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

#### 4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/

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# PATENT COOPERATION TREATY

## PCT

### INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference <b>27779/35932/PCT</b>	<div style="display: flex; justify-content: space-between;"> <div> <b>FOR FURTHER ACTION</b> </div> <div>           See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)         </div> </div>	
International application No. <b>PCT/US99/21591</b>	International filing date (day/month/year) <b>16/09/1999</b>	Priority date (day/month/year) <b>17/09/1998</b>
International Patent Classification (IPC) or national classification and IPC <b>A61K39/00</b>		
Applicant <b>ZONAGEN, INC. et al.</b>		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 13 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand  <b>13/04/2000</b>	Date of completion of this report  <b>15.01.2001</b>
Name and mailing address of the international preliminary examining authority:  <div style="display: flex; align-items: center;"> <div>             European Patent Office - P.B. 5818 Patentlaan 2              NL-2280 HV Rijswijk - Pays Bas              Tel. +31 70 340 - 2040 Tx: 31 651 epo nl              Fax: +31 70 340 - 3016           </div> </div>	Authorized officer  <b>Fernandez y Branas,F</b>  Telephone No. +31 70 340 2774



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/US99/21591

**I. Basis of the report**

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

**Description, pages:**

1-21 as originally filed

31-35 as received on 21/09/2000 with letter of 20/09/2000

**Claims, No.:**

1-58 as received on 21/09/2000 with letter of 20/09/2000

2. The amendments have resulted in the cancellation of:

☐ the description, pages:

☐ the claims, Nos.:

☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

Novelty (N)	Yes:	Claims	1-58
	No:	Claims	
Inventive step (IS)	Yes:	Claims	25-46
	No:	Claims	1-24, 47-58
Industrial applicability (IA)	Yes:	Claims	1-58
	No:	Claims	



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/US99/21591

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2. Citations and explanations

**see separate sheet**

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

**see separate sheet**

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/US99/21591

**Re Item V**

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

D1...IMMUNOLOGY TODAY, 15:469-474, 1994

D2....THE LANCET 1988, VOL 1 (8598) 1295-1298

D3....WO-A-9609805 (ZONAGEN, INC)

D1 discloses the existing birth-control vaccines containing hCG, such as hCG $\beta$ -TT conjugates adsorbed on AL(OH)<sub>3</sub> or hCG $\beta$ -CTP(109-145)-DT conjugates with MDP, see page 470 box 1.

Further details concerning the ranges of protein contained in the vaccines of D1 are provided in D2, see table I, page 1296. D2 is commented in D1, see reference 7 and hence can be considered to be included in the disclosure of D1.

In view of the prior art the subject matter of claims 1-58 appears to be new in the sense of Article 33(2) PCT.

For the analysis of the inventive step of the claims 1-58 D1 is considered to be the closest prior art. The main difference between the present application and D1 is that D1 uses

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/US99/21591

adjuvants other than chitosan. In view of this difference the problem to be solved by the present application can be defined as the provision of alternative vaccines comprising hCG $\beta$  and adjuvants for inducing antifertility.

D3 describes the adjuvant efficacy of chitosan to potentiate the immune response against antigens. D3 shows said effect with zona pellucida antigens in examples 11-12. While it could be stated that there is no direct suggestion in D3 or in D1 to the combination of chitosan and hCG $\beta$  to induce infertility by mounting an immune response against hCG $\beta$ , it is also the case that the present application only exemplifies that said combination induces an antibody response against hCG $\beta$  (see example 6). However, the technical problem to be solved as defined above ( provision of alternative vaccines comprising hCG $\beta$  and adjuvants for inducing antifertility) has not been shown to have actually been solved in the present application, as no evidence supporting the antifertility efficacy of the claimed vaccines has been provided.

The IPEA is of the opinion that what has been shown in example 6 of the present application (obtaining an antibody response against hCG $\beta$ ) would have been obvious for the skilled person on the basis of the teachings of D1 and D3 without any inventive skill, as the skilled person would have expected, taking into account the known adjuvant properties of chitosan (D3), that the combination chitosan-hCG $\beta$  would have induced an immune response against said hCG $\beta$ . Hence, the subject matter of claims 1-24 and 47-58 appears to lack an inventive step in the sense of Article 33(3) PCT.

On the contrary there is no suggestion in the prior art concerning the methods of claims 25-46 and thus this matter involves an inventive step in the sense of Article 33(3) PCT.

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/US99/21591

**Re Item VIII**

Certain observations on the international application

The terms "fragments" and "analogs" of claims 1, 13, 25, 36, and 47 are open-ended and as a result the scope of said claims is vaguely defined and thus said claims are considered as unclear in the sense of Article 6 PCT.

SEQUENCE LISTING

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AMENDED SHEET  
IPEA/EP

# AMENDED SHEET

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-33-

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AMENDED SHEET

-34-

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AMENDED SHEET  
 IPEA/EP



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AMENDED SHEET  
IPEA/EP

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**WE CLAIM:**

1. A composition comprising  $\beta$  human chorionic gonadotropin protein ( $\beta$ hCG) and/or fusions, fragments or analogs thereof,  
5 and a chitosan-based adjuvant, wherein the amount of  $\beta$ hCG ranges from about 10  $\mu$ g to about 500  $\mu$ g.
2. The composition of claim 1 wherein the amount of  $\beta$ hCG is about 25  $\mu$ g.  
10
3. The composition of claim 2 wherein the amount of  $\beta$ hCG is about 250  $\mu$ g.
4. The composition of claim 1-3 wherein the  $\beta$  human  
15 chorionic gonadotropin protein comprises a recombinant polypeptide.
5. The composition of claim 4 wherein the recombinant polypeptide further comprises the amino acid sequence of SEQ ID NO: 2 or  
4.  
20
6. The composition of claim 1 wherein the chitosan-based adjuvant comprises an emulsion of chitosan, sodium hydroxide, a biodegradable oil, a surfactant, and an aqueous buffer.
- 25 7. The composition of claim 6 wherein the biodegradable oil is squalene.
8. The composition of claim 6 wherein the ratio of  $\beta$ hCG protein and/or fusions, fragments or analogs thereof to adjuvant is in the  
30 range of about 1:20 (w/w) to about 1:1500 (w/w).

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IDEA/ED

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9. The composition of claim 1 wherein the adjuvant comprises chitosan, a metal salt, and an aqueous buffer.

5 10. The composition of claim 9 wherein the metal salt is selected from the group consisting of zinc acetate, nickel sulfate, and copper sulfate.

10 11. The composition of claim 9 wherein the ratio of  $\beta$ hCG, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).

15 12. The composition of claim 4 wherein the recombinant  $\beta$ hCG comprises a fusion protein consisting essentially of a  $\beta$ hCG protein or fragment or analog thereof joined to a  $\beta$ -galactosidase protein or fragment thereof.

20 13. A method of inducing infertility in a female mammal comprising administering at least one dose of a vaccine containing a  $\beta$ hCG proteins and/or fusions, fragments or analogs thereof in combination with a chitosan-based adjuvant in an amount effective to stimulate production of antibodies which recognize native circulating hCG proteins.

25 14. The method of claim 13 wherein the amount of  $\beta$ hCG ranges from about 10  $\mu$ g to about 500  $\mu$ g

15. The method of claim 14 wherein the amount of  $\beta$ hCG is about 250  $\mu$ g.

30 16. The method of claim 13-15 wherein the  $\beta$  human chorionic gonadotropin protein comprises a recombinant polypeptide.

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IPFA/EP

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17. The method of claim 16 wherein the recombinant polypeptide further comprises the amino acid sequence of SEQ ID NO: 2 or 4.

5 18. The method of claim 13 wherein the chitosan-based adjuvant comprises an emulsion of chitosan, sodium hydroxide, a biodegradable oil, a surfactant, and an aqueous buffer.

10 19. The method of claim 18 wherein the biodegradable oil is squalene.

20. The method of claim 18 wherein the ratio of  $\beta$ hCG protein and/or fusions, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).

15 21. The method of claim 13 wherein the adjuvant comprises chitosan, a metal salt, and an aqueous buffer.

20 22. The method of claim 21 wherein the metal salt is selected from the group consisting of zinc acetate, nickel sulfate, and copper sulfate.

25 23. The method of claim 21 wherein the ratio of  $\beta$ hCG protein and/or fusions, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).

30 24. The method of claim 16 wherein the recombinant  $\beta$ hCG comprises a fusion protein consisting essentially of a  $\beta$ hCG protein or fragment or analog thereof joined to a  $\beta$ -galactosidase protein or fragment thereof.

SUBSTITUTE SHEET

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105A/EP

25. A method for inducing transient infertility in a mammal comprising:

- 5 a) administering a recombinant  $\beta$ hCG protein and/or fusion, fragment or analog thereof expressed by a species of host cell in combination with a chitosan-based adjuvant; and
- 10 b) administering a recombinant  $\beta$ hCG protein, fragment or analog thereof, expressed by a different species of host cell than said recombinant  $\beta$ hCG administered in step a) in combination with a chitosan-based adjuvant; and

wherein the amount of  $\beta$ hCG administered in step b) is effective to stimulate production of antibodies which recognize native circulating hCG proteins.

15 26. The method of claim 25 wherein the amount of  $\beta$ hCG ranges from about 10  $\mu$ g to about 500  $\mu$ g.

27. The method of claim 26 wherein the amount of  $\beta$ hCG is about 250  $\mu$ g.

20 28. The method of claim 25 wherein the recombinant polypeptide further comprises the amino acid sequence of SEQ ID NO: 2 or 4.

25 29. The method of claim 25 wherein the chitosan-based adjuvant comprises an emulsion of chitosan, sodium hydroxide, a biodegradable oil, a surfactant, and an aqueous buffer.

30 30. The method of claim 29 wherein the biodegradable oil is squalene.

31. The method of claim 29 wherein the ratio of  $\beta$ hCG protein and/or fusions, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).

5 32. The method of claim 25 wherein the adjuvant comprises chitosan, a metal salt, and an aqueous buffer.

33. The method of claim 32 wherein the metal salt is selected from the group consisting of zinc acetate, nickel sulfate, and copper sulfate.  
10

34. The method of claim 32 wherein the ratio of  $\beta$ hCG proteins and/or fusions, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).  
15

35. The method of claim 25 wherein the recombinant  $\beta$ hCG comprises a fusion protein consisting essentially of a  $\beta$ hCG protein or fragment or analog thereof joined to a  $\beta$ -galactosidase protein or fragment thereof.  
20

36. A method of inducing antibody formation in a mammal comprising:

- 25
- a) administering a recombinant  $\beta$ hCG protein and/or fusion, fragment or analog thereof expressed by a species of host cell in combination with a chitosan-based adjuvant; and
  - b) administering a recombinant  $\beta$ hCG protein and/or fusion, fragment or analog thereof, expressed by a different species of host cell than said recombinant  $\beta$ hCG administered in step a) in combination with a chitosan-based adjuvant; and  
30

wherein the amount of  $\beta$ hCG administered in step b) is effective to stimulate production of antibodies which recognize native circulating hCG proteins.

5           37.    The method of claim 36 wherein the amount of  $\beta$ hCG ranges from about 10  $\mu$ g to about 500  $\mu$ g.

          38.    The method of claim 37 wherein the amount of  $\beta$ hCG is about 250  $\mu$ g.

10           39.    The method of claim 37 wherein the recombinant polypeptide further comprises the amino acid sequence of SEQ ID NO: 2 or 4.

          40.    The method of claim 36 wherein the chitosan-based  
15    adjuvant comprises an emulsion of chitosan, sodium hydroxide, a biodegradable oil, a surfactant, and an aqueous buffer.

          41.    The method of claim 40 wherein the biodegradable oil is  
20    squalene.

          42.    The method of claim 40 wherein the ratio of  $\beta$ hCG proteins and/or fusions, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).

25           43.    The method of claim 36 wherein the adjuvant comprises chitosan, a metal salt, and an aqueous buffer.

          44.    The method of claim 43 wherein the metal salt is  
30    selected from the group consisting of zinc acetate, nickel sulfate, and copper sulfate.

45. The method of claim 43 wherein the ratio of  $\beta$ hCG proteins and/or fusions, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).

46. The method of claim 36 wherein the recombinant  $\beta$ hCG comprises a fusion protein consisting essentially of a  $\beta$ hCG protein or fragment or analog thereof joined to a  $\beta$ -galactosidase protein or fragment thereof.

47. The use of  $\beta$ hCG for the manufacture of a medicament for inducing transient infertility in a mammal wherein the medicament comprises an injectable formulation containing  $\beta$  human chorionic gonadotropin proteins and/or fusions, fragments or analogs thereof, in combination with a chitosan-based adjuvant in an amount effective to stimulate production of antibodies which recognize native circulating hCG proteins.

48. The use of claim 47 wherein the amount of  $\beta$ hCG ranges from about 10  $\mu$ g to about 500  $\mu$ g.

49. The use of claim 48 wherein the amount of  $\beta$ hCG is about 250  $\mu$ g.

50. The use of claim 47 wherein the  $\beta$  human chorionic gonadotropin protein comprises a recombinant polypeptide.

51. The use of claim 50 wherein the recombinant polypeptide further comprises the amino acid sequence of SEQ ID NO: 2 or 4.



- 29 -

52. The use of claim 47 wherein the chitosan-based adjuvant comprises an emulsion of chitosan, sodium hydroxide, a biodegradable oil, a surfactant, and an aqueous buffer.

5 53. The use of claim 52 wherein the biodegradable oil is squalene.

54. The use of claim 52 wherein the ratio of  $\beta$ hCG protein, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to  
10 about 1:1500 (w/w).

55. The use of claim 47 wherein the adjuvant comprises chitosan, a metal salt, and an aqueous buffer.

15 56. The use of claim 55 wherein the metal salt is selected from the group consisting of zinc acetate, nickel sulfate, and copper sulfate.

57. The use of claim 55 wherein the ratio of  $\beta$ hCG proteins and/or fusions, fragments or analogs thereof to adjuvant is in the range of  
20 about 1:20 (w/w) to about 1:1500 (w/w).

58. The use of claim 50 wherein the recombinant  $\beta$ hCG comprises a fusion protein consisting essentially of a  $\beta$ hCG protein or fragment or analog thereof joined to a  $\beta$ -galactosidase protein or fragment  
25 thereof.

SUBSTITUTE SHEET

AMENDED SHEET

DEA/ED

# PATENT COOPERATION TREATY

## PCT

REC'D 15 JAN 2001

### INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference <b>27779/35932/PCT</b>	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) <b>FOR FURTHER ACTION</b>	
International application No. <b>PCT/US99/21591</b>	International filing date (day/month/year) <b>16/09/1999</b>	Priority date (day/month/year) <b>17/09/1998</b>
International Patent Classification (IPC) or national classification and IPC <b>A61K39/00</b>		
Applicant <b>ZONAGEN, INC. et al.</b>		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
  
2. This REPORT consists of a total of 6 sheets, including this cover sheet.
 

☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 13 sheets.

3. This report contains indications relating to the following items:

- I    ☒ Basis of the report
- II   ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV   ☐ Lack of unity of invention
- V    ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI   ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand  <b>13/04/2000</b>	Date of completion of this report  <b>15.01.2001</b>
Name and mailing address of the international preliminary examining authority:  European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized officer  <b>Fernandez y Branas, F</b>  Telephone No. +31 70 340 2774



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/US99/21591

**I. Basis of the report**

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

**Description, pages:**

1-21	as originally filed		
31-35	as received on	21/09/2000	with letter of 20/09/2000

**Claims, No.:**

1-58	as received on	21/09/2000	with letter of 20/09/2000
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2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:  
☐ the claims, Nos.:  
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

Novelty (N)	Yes: Claims 1-58
	No: Claims
Inventive step (IS)	Yes: Claims 25-46
	No: Claims 1-24, 47-58
Industrial applicability (IA)	Yes: Claims 1-58
	No: Claims

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/US99/21591

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2. Citations and explanations

**see separate sheet**

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

**see separate sheet**

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/US99/21591

**Re Item V**

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

D1...IMMUNOLOGY TODAY, 15:469-474, 1994

D2....THE LANCET 1988, VOL 1 (8598) 1295-1298

D3....WO-A-9609805 (ZONAGEN, INC)

D1 discloses the existing birth-control vaccines containing hCG, such as hCG $\beta$ -TT conjugates adsorbed on AL(OH)<sub>3</sub> or hCG $\beta$ -CTP(109-145)-DT conjugates with MDP, see page 470 box 1.

Further details concerning the ranges of protein contained in the vaccines of D1 are provided in D2, see table I, page 1296. D2 is commented in D1, see reference 7 and hence can be considered to be included in the disclosure of D1.

In view of the prior art the subject matter of claims 1-58 appears to be new in the sense of Article 33(2) PCT.

For the analysis of the inventive step of the claims 1-58 D1 is considered to be the closest prior art. The main difference between the present application and D1 is that D1 uses

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/US99/21591

adjuvants other than chitosan. In view of this difference the problem to be solved by the present application can be defined as the provision of alternative vaccines comprising hCG $\beta$  and adjuvants for inducing antifertility.

D3 describes the adjuvant efficacy of chitosan to potentiate the immune response against antigens. D3 shows said effect with zona pellucida antigens in examples 11-12. While it could be stated that there is no direct suggestion in D3 or in D1 to the combination of chitosan and hCG $\beta$  to induce infertility by mounting an immune response against hCG $\beta$ , it is also the case that the present application only exemplifies that said combination induces an antibody response against hCG $\beta$  (see example 6). However, the technical problem to be solved as defined above ( provision of alternative vaccines comprising hCG $\beta$  and adjuvants for inducing antifertility) has not been shown to have actually been solved in the present application, as no evidence supporting the antifertility efficacy of the claimed vaccines has been provided.

The IPEA is of the opinion that what has been shown in example 6 of the present application (obtaining an antibody response against hCG $\beta$ ) would have been obvious for the skilled person on the basis of the teachings of D1 and D3 without any inventive skill, as the skilled person would have expected, taking into account the known adjuvant properties of chitosan (D3), that the combination chitosan-hCG $\beta$  would have induced an immune response against said hCG $\beta$ . Hence, the subject matter of claims 1-24 and 47-58 appears to lack an inventive step in the sense of Article 33(3) PCT.

On the contrary there is no suggestion in the prior art concerning the methods of claims 25-46 and thus this matter involves an inventive step in the sense of Article 33(3) PCT.

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/US99/21591

**Re Item VIII**

Certain observations on the international application

The terms "fragments" and "analogs" of claims 1, 13, 25, 36, and 47 are open-ended and as a result the scope of said claims is vaguely defined and thus said claims are considered as unclear in the sense of Article 6 PCT.

- 1 -

## SEQUENCE LISTING

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&lt;120&gt; HUMAN CHORIONIC GONADOTROPIN

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- 5 -

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**WE CLAIM:**

1. A composition comprising  $\beta$  human chorionic gonadotropin protein ( $\beta$ hCG) and/or fusions, fragments or analogs thereof, and a chitosan-based adjuvant.

5 2. The composition of claim 1 wherein the amount of  $\beta$ hCG ranges from about 10  $\mu$ g to about 500  $\mu$ g.

3. The composition of claim 2 wherein the amount of  $\beta$ hCG is about 250  $\mu$ g.

10 4. The composition of claim 1-3 wherein the  $\beta$  human chorionic gonadotropin protein comprises a recombinant polypeptide.

5. The composition of claim 4 wherein the recombinant polypeptide further comprises the amino acid sequence of SEQ ID NO: 2 or 4.

15 6. The composition of claim 1 wherein the chitosan-based adjuvant comprises an emulsion of chitosan, sodium hydroxide, a biodegradable oil, a surfactant, and an aqueous buffer.

7. The composition of claim 6 wherein the biodegradable oil is squalene.

20 8. The composition of claim 6 wherein the ratio of  $\beta$ hCG protein and/or fusions, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).

9. The composition of claim 1 wherein the adjuvant comprises chitosan, a metal salt, and an aqueous buffer.

10. The composition of claim 9 wherein the metal salt is selected from the group consisting of zinc acetate, nickel sulfate, and copper sulfate.

5 11. The composition of claim 9 wherein the ratio of  $\beta$ hCG, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).

10 12. The composition of claim 4 wherein the recombinant  $\beta$ hCG comprises a fusion protein consisting essentially of a  $\beta$ hCG protein or fragment or analog thereof joined to a  $\beta$ -galactosidase protein or fragment thereof.

15 13. A method of inducing infertility in a female mammal comprising administering at least one dose of a vaccine containing a  $\beta$ hCG proteins and/or fusions, fragments or analogs thereof optionally in combination with a chitosan-based adjuvant in an amount effective to stimulate production of antibodies which recognize native circulating hCG proteins.

14. The method of claim 13 wherein the amount of  $\beta$ hCG ranges from about 10  $\mu$ g to about 500  $\mu$ g.

20 15. The method of claim 14 wherein the amount of  $\beta$ hCG is about 250  $\mu$ g.

16. The method of claim 13-15 wherein the  $\beta$  human chorionic gonadotropin protein comprises a recombinant polypeptide.

17. The method of claim 16 wherein the recombinant polypeptide further comprises the amino acid sequence of SEQ ID NO: 2 or 4.

18. The method of claim 13 wherein the chitosan-based adjuvant comprises an emulsion of chitosan, sodium hydroxide, a biodegradable oil, a surfactant, and an aqueous buffer.

19. The method of claim 18 wherein the biodegradable oil is squalene.

20. The method of claim 18 wherein the ratio of  $\beta$ hCG protein and/or fusions, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).

21. The method of claim 13 wherein the adjuvant comprises chitosan, a metal salt, and an aqueous buffer.

22. The method of claim 21 wherein the metal salt is selected from the group consisting of zinc acetate, nickel sulfate, and copper sulfate.

23. The method of claim 21 wherein the ratio of  $\beta$ hCG protein and/or fusions, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).

24. The method of claim 16 wherein the recombinant  $\beta$ hCG comprises a fusion protein consisting essentially of a  $\beta$ hCG protein or fragment or analog thereof joined to a  $\beta$ -galactosidase protein or fragment thereof.

25. A method for inducing transient infertility in a mammal comprising:

- 5 a) administering a recombinant  $\beta$ hCG protein and/or fusion, fragment or analog thereof expressed by a species of host cell optionally in combination with a chitosan-based adjuvant; and
- b) administering a recombinant  $\beta$ hCG protein, fragment or analog thereof, expressed by a different species of host cell than said recombinant  $\beta$ hCG administered in step a) optionally in combination with a chitosan-based adjuvant; and

10 wherein the amount of  $\beta$ hCG administered in step b) is effective to stimulate production of antibodies which recognize native circulating hCG proteins.

26. The method of claim 25 wherein the amount of  $\beta$ hCG ranges from about 10  $\mu$ g to about 500  $\mu$ g.

15 27. The method of claim 26 wherein the amount of  $\beta$ hCG is about 250  $\mu$ g.

28. The method of claim 25 wherein the recombinant polypeptide further comprises the amino acid sequence of SEQ ID NO: 2 or 4.

20 29. The method of claim 25 wherein the chitosan-based adjuvant comprises an emulsion of chitosan, sodium hydroxide, a biodegradable oil, a surfactant, and an aqueous buffer.

30. The method of claim 29 wherein the biodegradable oil is squalene.

25 31. The method of claim 29 wherein the ratio of  $\beta$ hCG protein and/or fusions, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).



32. The method of claim 25 wherein the adjuvant comprises chitosan, a metal salt, and an aqueous buffer.

33. The method of claim 32 wherein the metal salt is selected from the group consisting of zinc acetate, nickel sulfate, and copper sulfate.

5                   34. The method of claim 32 wherein the ratio of  $\beta$ hCG proteins and/or fusions, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).

10                   35. The method of claim 25 wherein the recombinant  $\beta$ hCG comprises a fusion protein consisting essentially of a  $\beta$ hCG protein or fragment or analog thereof joined to a  $\beta$ -galactosidase protein or fragment thereof.

36. A method of inducing antibody formation in a mammal comprising:

15                   a) administering a recombinant  $\beta$ hCG protein and/or fusion, fragment or analog thereof expressed by a species of host cell optionally in combination with a chitosan-based adjuvant; and  
b) administering a recombinant  $\beta$ hCG protein and/or fusion, fragment or analog thereof, expressed by a different species of host cell than said recombinant  $\beta$ hCG administered in step a)  
20                   optionally in combination with a chitosan-based adjuvant; and wherein the amount of  $\beta$ hCG administered in step b) is effective to stimulate production of antibodies which recognize native circulating hCG proteins.

37. The method of claim 36 wherein the amount of  $\beta$ hCG ranges from about 10  $\mu$ g to about 500  $\mu$ g.

38. The method of claim 37 wherein the amount of  $\beta$ hCG is about 250  $\mu$ g.

39. The method of claim 37 wherein the recombinant polypeptide further comprises the amino acid sequence of SEQ ID NO: 2 or 4.

5                   40. The method of claim 36 wherein the chitosan-based adjuvant comprises an emulsion of chitosan, sodium hydroxide, a biodegradable oil, a surfactant, and an aqueous buffer.

41. The method of claim 40 wherein the biodegradable oil is squalene.

10                   42. The method of claim 40 wherein the ratio of  $\beta$ hCG proteins and/or fusions, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).

43. The method of claim 36 wherein the adjuvant comprises chitosan, a metal salt, and an aqueous buffer.

15                   44. The method of claim 43 wherein the metal salt is selected from the group consisting of zinc acetate, nickel sulfate, and copper sulfate.

45. The method of claim 43 wherein the ratio of  $\beta$ hCG proteins and/or fusions, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).

20                   46. The method of claim 36 wherein the recombinant  $\beta$ hCG comprises a fusion protein consisting essentially of a  $\beta$ hCG protein or fragment or analog thereof joined to a  $\beta$ -galactosidase protein or fragment thereof.

5                   47.     The use of  $\beta$ hCG for the manufacture of a medicament for inducing transient infertility in a mammal wherein the medicament comprises an injectable formulation containing  $\beta$  human chorionic gonadotropin proteins and/or fusions, fragments or analogs thereof, optionally in combination with a chitosan-based adjuvant in an amount effective to stimulate production of antibodies which recognize native circulating hCG proteins.

                  48.     The use of claim 47 wherein the amount of  $\beta$ hCG ranges from about 10  $\mu$ g to about 500  $\mu$ g.

10                  49.     The use of claim 48 wherein the amount of  $\beta$ hCG is about 250  $\mu$ g.

                  50.     The use of claim 47 wherein the  $\beta$  human chorionic gonadotropin protein comprises a recombinant polypeptide.

                  51.     The use of claim 50 wherein the recombinant polypeptide further comprises the amino acid sequence of SEQ ID NO: 2 or 4.

15                  52.     The use of claim 47 wherein the chitosan-based adjuvant comprises an emulsion of chitosan, sodium hydroxide, a biodegradable oil, a surfactant, and an aqueous buffer.

                  53.     The use of claim 52 wherein the biodegradable oil is squalene.

20                  54.     The use of claim 52 wherein the ratio of  $\beta$ hCG protein, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).

55. The use of claim 47 wherein the adjuvant comprises chitosan, a metal salt, and an aqueous buffer.

56. The use of claim 55 wherein the metal salt is selected from the group consisting of zinc acetate, nickel sulfate, and copper sulfate.

5 57. The use of claim 55 wherein the ratio of  $\beta$ hCG proteins and/or fusions, fragments or analogs thereof to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w).

10 58. The use of claim 50 wherein the recombinant  $\beta$ hCG comprises a fusion protein consisting essentially of a  $\beta$ hCG protein or fragment or analog thereof joined to a  $\beta$ -galactosidase protein or fragment thereof.

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# 基因工程菌株产生含人类绒毛膜促性腺激素抗原的研究

## II. $\beta$ -galactosidase-hCG 聚体蛋白免疫原性

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### 摘要

基因工程菌株产生的含人类绒毛膜促性腺激素(hCG)  $\beta$ 亚单位C末端36肽的 $\beta$ -半乳糖苷酶杂交蛋白(简称 $\beta$ -galactosidase-hCG),在未经解聚处理前免疫新西兰种白兔和昆明种小白鼠,其抗血清在免疫双扩散试验和对流免疫电泳中对hCG没有形成沉淀线作用。而此杂交蛋白经解聚处理后,其抗血清与hCG相作用,在对流免疫电泳和免疫双扩散试验中都出现抗原抗体反应的免疫沉淀线。从多种蛋白质解聚系统中,经过反复试验,筛选出对 $\beta$ -galactosidase-hCG杂交蛋白有良好解聚作用的解聚剂为2 M脲、0.9 M SDS、0.005 M 巯基乙醇;解聚作用最适温度是60℃,最适时间为40分钟。

关键词:  $\beta$ -hCG C末端36肽;  $\beta$ -半乳糖苷酶; 巯基乙醇; SDS免疫原性

### 1 前言

在研究人类绒毛膜促性腺激素 $\beta$ 亚单位C末端多肽片段( $\beta$ -hCG C-terminal peptide)为抗原的避孕疫苗中,我们通过基因工程,用寡核苷酸合成仪合成了hCG  $\beta$ 亚单位C末端36肽基因,成功地克隆到含 $\beta$ -半乳糖苷酶基因的噬菌体载体 $\lambda$ gt11中,并在宿主菌E. Coli Y1089中得到表达<sup>1</sup>。 $\beta$ -半乳糖苷酶基因在大肠杆菌和酵母中的表达,常用作基因融合是否成功和基因调节状况的分析<sup>2</sup>。 $\beta$ -半乳糖苷酶常以四聚体形式存在,分子量为540 000道尔顿<sup>3</sup>,除四聚体外,随所处环境条件不同,可聚合或解聚成“二聚体”、“寡聚体”或“单体”。每个单体分子量为135 000道尔顿,肽链呈螺旋状蜿蜒盘曲<sup>4</sup>。从 $\lambda$ gt11hCG Y1089菌体中分离纯化得到的含hCG  $\beta$ 亚单位C末端36肽的 $\beta$ -半乳糖苷酶杂交蛋白(下简称杂交蛋白)未经解聚处理免疫新西兰种雄兔和昆明种雄小白鼠,其抗血清与杂交蛋白相作用,不论在对流免疫或免疫双扩散试验中都出现明显的抗原抗体反应沉淀线,说明杂交蛋白有强的免疫原性。然而,将此抗血清与hCG相作用,则没有抗原抗体反应沉淀线的形成,这表明克隆基因

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表达的hCG $\beta$ 亚单位C末端36肽未能作为抗原决定簇刺激机体产生相应抗体。对增强蛋白质免疫原性,直至目前仍多从蛋白质空间结构与抗原决定簇裸露方面考虑,于是我们采用蛋白质解聚剂氯乙醇<sup>6</sup>、脲<sup>7</sup>和甲酰胺<sup>7</sup>等试剂,在一定条件下对此杂交蛋白进行解聚,使杂交蛋白在一级结构不改变的前提下,空间的构型有所改变,令hCG $\beta$ 亚单位C端36肽抗原决定簇裸露,能在有机体内显示其免疫原性。试验结果,杂交蛋白经解聚处理后,其抗血清在对流免疫和免疫双扩散试验中与hCG都出现抗原抗体反应的沉淀线,表明解聚处理后,杂交蛋白中hCG $\beta$ 亚单位C末端36肽的抗原决定簇刺激机体产生了相应的抗体。脲对蛋白质来说是一种变性剂,随着温度、pH值和盐浓度的变化<sup>8</sup>或其本身浓度不同<sup>9</sup>,可使蛋白质有不同程度的解聚或变性作用,为此,我们对不同解聚剂的解聚效果进行比较研究,对解聚的合适条件亦进行了探讨。

## 2 材料与方法

### 2.1 材料

纯化 $\beta$ -半乳糖苷酶杂交蛋白(本研究室自制)。

标准 $\beta$ -半乳糖苷酶(美国Boehringer产品)、十二烷基硫酸钠(SDS),巯基乙醇(Sigma,公司产品)、考马斯亮兰(Fluka厂生产)、甲撑双丙烯酰胺(Bis)、TEMED和Pyronin Y(G)(Bio-RAD产品)、完全和不完全弗氏佐剂(GIBC产品)、脲、氯乙醇和甲酰胺(国产化学纯试剂)、丙烯酰胺、琼脂糖等(国产分析纯试剂)。

根据氯乙醇、甲酰胺、SDS和脲等对蛋白质来说都是变性剂,在不同条件下对蛋白质有不同程度解聚和变性作用,因而把这些试剂组成四个配方:

I. 67%氯乙醇、0.9M SDS、0.005M巯基乙醇。

II. 67%甲酰胺、0.9M SDS、0.005M巯基乙醇。

III. 68% N,N-二甲基酰胺、0.9M SDS、0.005M巯基乙醇。

IV. 2M脲、0.9M SDS、0.005M巯基乙醇。

在相同条件下,对杂交蛋白进行解聚作用,并比较其解聚效果。

### 2.2 方法

2.2.1 最适解聚温度试验 将杂交蛋白50 $\mu$ l (5.8mg/ml)与等量配方IV解聚剂在30、40、50、60、70、80、90和100 $^{\circ}$ C中进行解聚处理10分钟,在10%聚丙烯酰胺凝胶电泳和免疫双扩散试验中检测解聚效果,选取合适解聚温度。

2.2.2 最适解聚时间测定 将50.1 (5.8mg/ml)杂交蛋白与等量解聚剂IV,在60 $^{\circ}$ C作用5、10、20、30、40、50和60分钟,然后用10%聚丙烯酰胺凝胶电泳和免疫双扩散测定解聚效果,选取最佳解聚时间。

2.2.3 免疫方法参照Weir<sup>10</sup>方法稍改 昆明种小白雄鼠体重平均为20克,每只平均用20微克杂交蛋白,第一次用完全弗氏佐剂,第二、三、四次用不完全弗氏佐剂,注射后肢内外侧肌肉,每次免疫时间相隔7天,在最后一次免疫后7天处死取血,用常规方法分离提取血清。

2.2.4 电泳分析解聚的杂交蛋白 杂交蛋白在一定条件下经解聚剂处理后,分别用10%聚丙烯酰胺凝胶垂直板电泳,按Laemmli<sup>11</sup>方法和圆盘电泳按Davis<sup>12</sup>方法进行电泳分析。样品在电泳前经90 $^{\circ}$ C加热10分钟处理,电泳缓冲液是含0.1%SDS的0.025M三羟甲基氨基甲烷(Tris)、0.192M甘氨酸, pH8.3的缓冲系统。垂直型板电泳、电压150V,圆盘

电泳电流每管 4 mA, 电泳 4~5 小时, 凝胶均用考马斯亮兰 R250 染色。

2.2.5. 对流免疫电泳和免疫双扩散 方法按前文<sup>1</sup>进行。琼脂糖浓度为 1%, 抗原是解聚和未解聚杂交蛋白以及纯 hCG, 抗体分别用鼠抗解聚与未解聚杂交蛋白抗血清, 凝胶用氨基黑 10B 染色。

### 3 结果与讨论

#### 3.1 几种解聚剂解聚效应的比较

以不同配方的四组解聚剂对含 hCG  $\beta$  亚单位 C 末端 36 肽的  $\beta$ -半乳糖苷酶在相同条件下进行解聚作用, 结果以第 IV 组试剂解聚效果最好, 见图 1 和图 2。

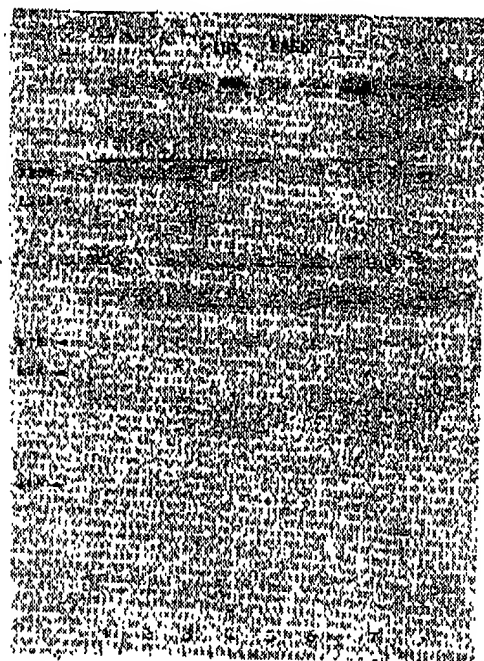


图 1 不同解聚剂对杂交蛋白有不同解聚效果

1. 高分子蛋白质标准分子量: 甲状腺球蛋白 (330 000), 铁蛋白 (220 000), 清蛋白 (67 000), 过氧化氢酶 (60 000), 乳酸脱氢酶 (36 000)
2.  $\beta$ -半乳糖苷酶
3. 未经解聚处理的杂交蛋白
4. 用解聚剂 IV 处理的杂交蛋白
- 5、6、7. 分别为用解聚剂 III、IV、I、I 解聚处理的杂交蛋白

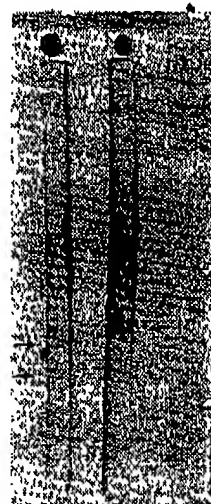


图 2 含 hCG  $\beta$  链 C 端 36 肽杂交蛋白圆盘电泳图谱

1. 未经解聚剂处理的杂交蛋白;
2. 经配方 IV 解聚剂处理的杂交蛋白

在板电泳 (图 1 中箭头指示) 经配方 IV 解聚剂处理的杂交蛋白上端出现少量不能进入凝胶的大分子聚体, 末端又有新的小分子出现, 其余区带与其他解聚剂处理和未经处理的杂交蛋白相比分子量都较小, 且区带呈波纹状, 这说明配方 IV 解聚剂使杂交蛋白聚集体所含单体数起变化和空间构型有所改变。而在圆盘电泳 (图 2) 中, 经配方 IV 解聚剂处理的除顶端不能进入凝胶的大分子, 在取胶时已脱落外还有二条区带, 而用其他组解聚剂处理和未经解聚的杂

交蛋白一样只有一条区带,表明配方Ⅳ解聚剂改变了杂交蛋白的结构。原因可能是配方Ⅳ中脲和SDS对杂交蛋白起到相辅相成的作用。这样,杂交蛋白在解聚或新聚体形成过程中一级结构不致改变而立体空间构型则起变化,导致杂交蛋白中所含hCG  $\beta$ 链C端36肽有裸露的可能。而氯乙醇既可以破坏疏水键,又有利于氢键的形成,使亚基的螺旋度增加,易促使大分子聚体的形成,于是在图1(7)上端积有较多不能进入凝胶的大分子,结果凝胶内区带较少;用甲酰胺处理的杂交蛋白,从图1(5)、(6)行可看出蛋白质分子没起什么变化。所以配方Ⅳ是较理想的解聚剂。

### 3.2 免疫原性的鉴定

将解聚前后的杂交蛋白分别免疫新西兰种雄兔和昆明种雄小白鼠,以其抗血清对解聚与未解聚杂交蛋白和hCG作对流免疫电泳以及免疫双扩散试验,结果是:

(1) 未解聚杂交蛋白的抗血清只与未解聚杂交蛋白有沉淀线形成,与解聚杂交蛋白和hCG无沉淀线形成(图3、4)。

(2) 解聚杂交蛋白抗血清与解聚杂交蛋白和hCG都出现抗原抗体反应沉淀线,与未解聚杂交蛋白则无反应(图5和图6)。

上述抗原抗体反应沉淀线的形成与否,说明经解聚后的杂交蛋白一级结构没有改变而空间结构则起了变化,hCG  $\beta$ -链C端36肽抗原决定簇有所暴露,刺激机体产生了相应抗体,不过其反应沉淀线较弱,形成沉淀线的时间需较长,且抗体滴度也较低,原因可能是由于36肽相对于分子量约140 000的杂交蛋白来说只是一个短的肽段,抗原决定簇相应较少,刺激机体所形成的抗体量少。因而抗原抗体反应沉淀线较弱。

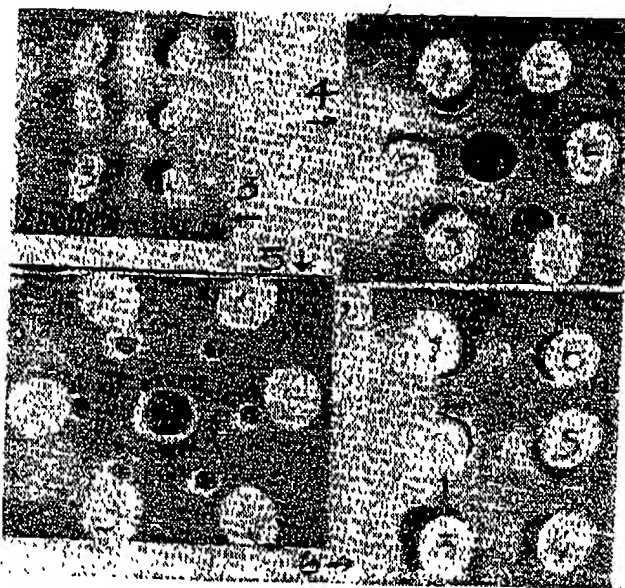


图3 对流免疫电泳

左边孔为抗原: 1. 未解聚杂交蛋白; 2. 解聚杂交蛋白; 3. hCG

右边孔为抗体: 4. 5. 6孔都是兔抗未解聚杂交蛋白抗血清。

图4 免疫双扩散

中央孔为抗体: 鼠抗未解聚杂交蛋白抗血清

孔1. 未解聚杂交蛋白; 2. 解聚杂交蛋白; 3. hCG

图5 对流免疫电泳

左边为抗原: 1. 解聚杂交蛋白; 2. hCG; 3. 未解聚杂交蛋白

右边为抗体: 4. 5. 6孔为鼠抗解聚杂交蛋白抗血清

图6 免疫双扩散

中央孔为抗体: 鼠抗解聚杂交蛋白抗血清

孔1、2为解聚杂交蛋白; 3、4为hCG; 5、6为未解聚杂交蛋白



### 3.3 各种解聚条件的比较

**3.3.1 不同温度对解聚效应的影响** 在时间固定为10分钟,用解聚剂Ⅳ在30~100℃范围内,每隔10℃处理杂交蛋白,结果从电泳图谱上下两端蛋白区带的变化,可看出60℃开始有新区带出现,(图7)80℃时最明显,90℃至100℃时慢慢减弱,在免疫双扩散试验中,以鼠抗解聚杂交蛋白的抗血清为抗体,不同温度处理的杂交蛋白为抗原,60℃处理的杂交蛋白所形成的沉淀线粗长而清晰,说明60℃处理形成的解聚蛋白量多且抗原性好。80℃处理的杂交蛋白在电泳图谱上区带较明显,然而在双扩散中形成的沉淀线反而较弱,且80℃高温易导致蛋白质生物活性丧失,不宜采用,所以宜选择60℃为解聚温度。

**3.3.2 解聚最佳时间的选择** 在固定60℃,用配方Ⅳ解聚剂对杂交蛋白以5~60分钟进行处理,从第5分钟取第1管,第10分钟取第2管,以后每间隔10分钟取1管,直至60分钟,电泳检查结果见图8。从图8可看出,第30分钟处理那一管的上下端开始出现新区带,40分钟最明显;而50和60分钟的反而依次减弱。在免疫双扩散试验中,以40分钟解聚的杂交蛋白与鼠抗解聚杂交蛋白抗血清,形成的沉淀线最强而清晰,30和50分钟次之,20和60分钟的更弱。由此可见,用解聚剂Ⅳ在60℃时对杂交蛋白进行解聚作用以40分钟最合适。

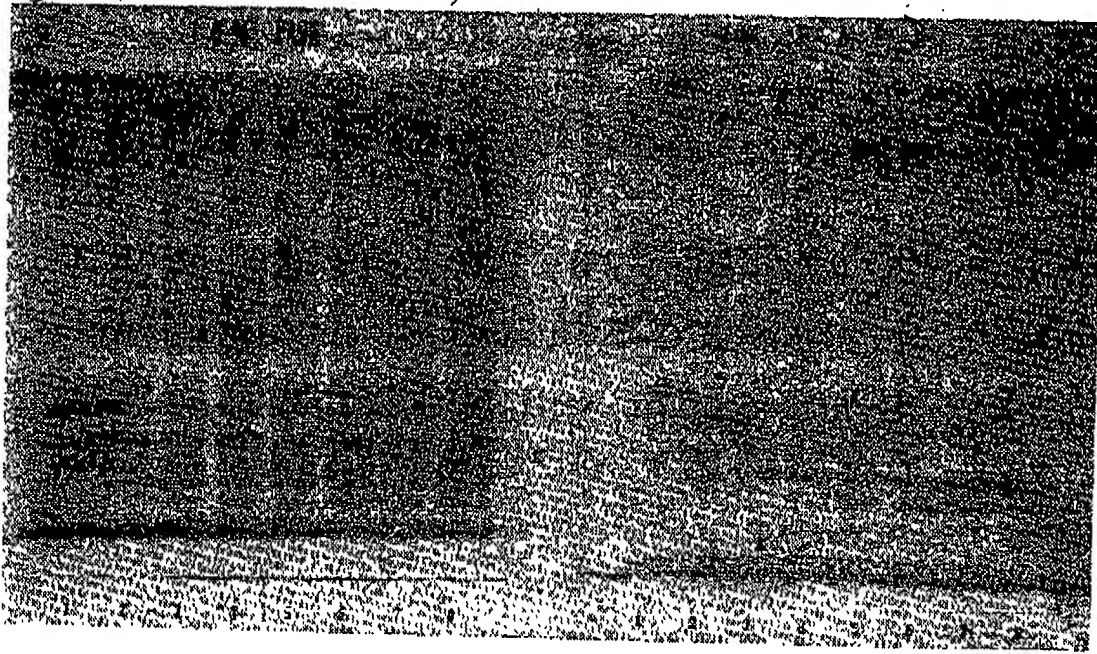


图7 解聚剂对杂交蛋白作用最适温度  
编号 1、2、3、4、5、6、7、  
8 分别代表用30、40、50、60、70、80、  
90和100℃处理的杂交蛋白。

图8 解聚剂对杂交蛋白作用最适时间  
编号 1、2、3、4、5、6、7、8  
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综合上述实验结果可看出,基因工程菌株产生的含hCG $\beta$ 链C端36肽的 $\beta$ -半乳糖苷酶杂交蛋白,经解聚剂Ⅳ处理后,从对hCG无中和免疫活性至产生中和免疫活性,说明此解聚剂对杂交蛋白的处理是成功的。60℃、40分钟是对此杂交蛋白的最佳解聚条件。解聚前后此 $\beta$ -半乳糖苷酶杂交蛋白结构变化与功能之间的相互关系,有待进一步深入研究。

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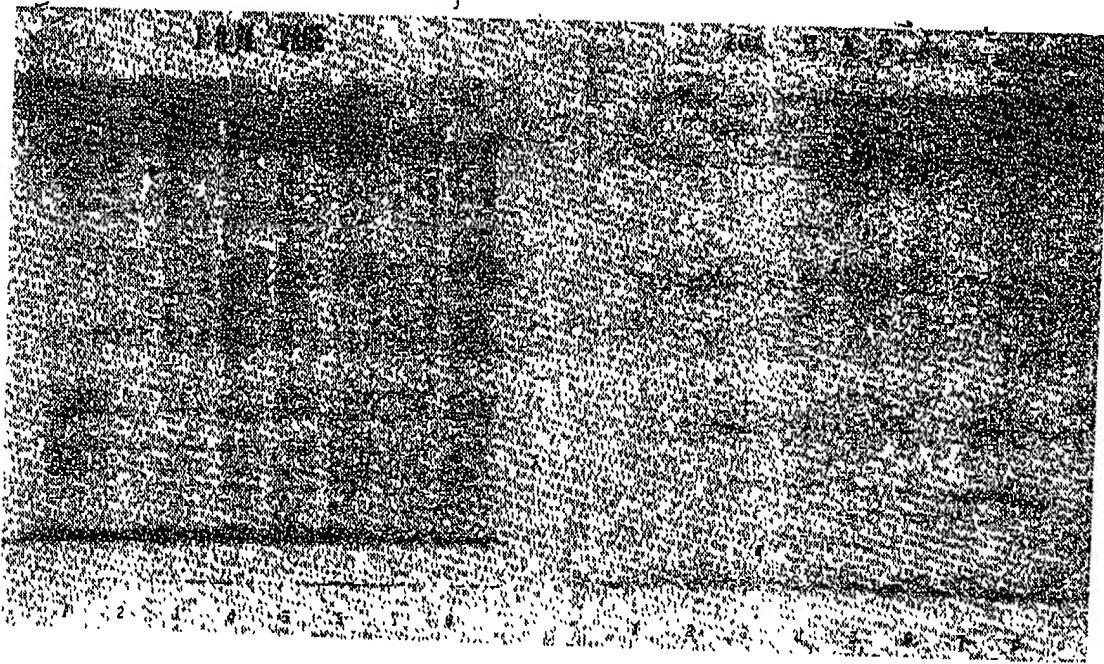


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编号 1、2、3、4、5、6、7、8  
分别代表未经处理的和经5、10、20、30、40、50、60分钟处理的杂交蛋白。

综合上述实验结果可看出,基因工程菌株产生的含hCGβ链C端36肽的β-半乳糖苷酶杂交蛋白,经解聚剂Ⅳ处理后,从对hCG无中和免疫活性至产生中和免疫活性,说明此解聚剂对杂交蛋白的处理是成功的。60℃、40分钟是对此杂交蛋白的最佳解聚条件。解聚前后此β-半乳糖苷酶杂交蛋白结构变化与功能之间的相互关系,有待进一步深入研究。

刘学高教授在工作中给予关怀和指导; 本研究课题得到世界卫生组织和广州市科委的赞助和支持。特此一并致谢!

### 参 考 文 献

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## Study on the Immunogenicity of $\beta$ -galactosidase-hCG Polymer Containing hCG- $\beta$ -subunit C-terminal Peptide Synthesized in $\lambda$ gt11 hCG Y1089

Luo Yuxiang, Liu Jieshen

Wu Boliang, Wu Xiaying, Wang Xuan

(Center for Reproductive

(Dept. of Biology)

Immunology Research)

### ABSTRACT

An artificial gene equivalent to 36 C-terminal peptide of human chorionic gonadotropin (hCG)  $\beta$ -subunit has been chemically synthesized through the application oligonucleotide synthesizer and the double strand DNA has been successfully cloned into an expression vector,  $\lambda$ gt11, and transfected into the host strain E.Coli Y1089, through recombinant phage  $\lambda$ gt11 hCG to produce a hybrid protein containing hCG- $\beta$ -subunit 36 C-terminal peptide (Called  $\beta$ -galactosidase-hCG). Anti-serum of rat immunized with  $\beta$ -galactosidase-hCG was detected by counter current electrophoresis and immunodiffusion. the results have clearly demonstrated that  $\beta$ -galactosidase-

hCG can be crossly immunoprecipitated with rat anti- $\beta$ -galactosidase-hCG serum, whereas using hCG replaced  $\beta$ -galactosidase-hCG, the anti-serum produced against  $\beta$ -galactosidase-hCG has no cross reaction with hCG. When  $\beta$ -galactosidase-hCG was treated with 2M Urea, 0.9M SDS and 0.005M Mercaptoethanol solvent system before using as an antigen to immunize rat, the anti-body against this depolymerized antigen can be crossly immunoprecipitated with hCG. It revealed that the  $\beta$ -galactosidase-hCG polymer was depolymerized and the conformation of  $\beta$ -galactosidase-hCG subunit has been changed, therefore the antigenic determinant of hCG- $\beta$ -subunit 36 C-terminal peptide in  $\beta$ -galactosidase-hCG appeared its immunogenicity in Urea and SDS solvent. the depolymerized optimal conditions for  $\beta$ -galactosidase-hCG in Urea and SDS solvent system were 60°C in 40 minutes.

KEYWORDS:  $\beta$ -hCG C-terminal peptide  $\beta$ -Galactosidase, Urea, Dodecyl sodium sulfate, Mercaptoethanol, Immunogenicity

(上接第36页)

## Indirect Potentiometric Stripping Analysis of Scandium

Yang Peihui, Li Deyu

(Dept. of Chemistry)

### ABSTRACT

An indirect potentiometric stripping analysis based on the difference in stability between two coordination compounds Sc-EDTA and Cd-EDTA was applied to the determination of Sc (III) in 1.2M Sodium chloride solution at pH 5.2. The sensitivity of this method is  $2 \times 10^{-8} M$  when dissolved oxygen ( $4.9 \times 10^{-5} M$ ) is used as oxidant at a plating potential of -0.9V for 2 minutes. The recovery of this method is 99.8%. This method may be used for the determination of trace Sc (III) in the mixed solution of rare earths.

KEYWORDS: Scandium, Potentiometric stripping analysis

## PATENT COOPERATION TREATY

RECEIVED

MAR 3 2000

From the INTERNATIONAL SEARCHING AUTHORITY

PCT MARSHALL C'TOOL

To:

MARSHALL, O'TOOLE, GERSTEIN,  
MURRAY & BORUN  
Attn. Uhl, J111 E.  
6300 Sears Tower  
233 South Wacker Drive  
Chicago, Illinois 60606-6402  
UNITED STATES OF AMERICA

NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL SEARCH REPORT  
OR THE DECLARATION

(PCT Rule 44.1)

Date of mailing  
(day/month/year)

23/02/2000

Applicant's or agent's file reference

27779/35932/PCT

FOR FURTHER ACTION

See paragraphs 1 and 4 below

International application No.

PCT/US 99/21591

International filing date  
(day/month/year)

16/09/1999

Applicant

ZONAGEN, INC. et al.

DOCKETED: 4/23/00

1. ☒ The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland  
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. Further action(s): The applicant is reminded of the following:

Shortly after 18 months from the priority date, the International application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the International application, or of the priority claim, must reach the International Bureau as provided in Rules 90b/s.1 and 90b/s.3, respectively, before the completion of the technical preparations for International publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority



European Patent Office, P.B. 5818 Patentlaan 2  
NL-2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Nina Vercio

## NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

### INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

#### What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

#### When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

#### Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

#### How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

#### What documents must/may accompany the amendments?

##### Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:  
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:  
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:  
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or  
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:  
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

**"Statement under article 19(1)" (Rule 46.4)**

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

**It must be in the language in which the international application is to be published.**

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

**Consequence if a demand for international preliminary examination has already been filed**

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

**Consequence with regard to translation of the international application for entry into the national phase**

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>27779/35932/PCT</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, Item 5 below.	
International application No. <b>PCT/US 99/ 21591</b>	International filing date (day/month/year) <b>16/09/1999</b>	(Earliest) Priority Date (day/month/year) <b>17/09/1998</b>
Applicant <b>ZONAGEN, INC. et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

## 1. Basis of the report

- a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing:

☒ contained in the international application in written form.

☒ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2. ☐ Certain claims were found unsearchable (See Box I).

3. ☐ Unity of invention is lacking (see Box II).

## 4. With regard to the title,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

## 5. With regard to the abstract,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

## 6. The figure of the drawings to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☐ None of the figures.



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/21591

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 7 A61K39/00 A61K39/39 //C07K14/59

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 368 253 A (UNION CARBIDE CHEM PLASTIC) 16 May 1990 (1990-05-16) column 2, line 32 - line 47 column 10, line 37 ---	1
X	DIRNHOFER STEPHAN: "The suitability of human chorionic gonadotropin (hCG)-based birth-control vaccines" IMMUNOLOGY TODAY, vol. 15, 1994, pages 469-474, XP002129457 cited in the application the whole document --- -/--	13-17, 47-51, 58

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

2 February 2000

Date of mailing of the international search report

23/02/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
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 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax: (+31-70) 340-3016

Authorized officer

Fernandez y Branas, F

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/21591

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JONE W.R. ET AL: "PHASE 1 CLINICAL TRIAL OF A WORLD HEALTH ORGANISATION BIRTH CONTROL VACCINE" THE LANCET, vol. I, no. 8598, 1988, pages 1295-1298, XP002129534 cited in the application the whole document	13-17, 47-51, 58
A	US 4 474 756 A (MITSUHASHI MASAKAZU ET AL) 2 October 1984 (1984-10-02) the whole document	1-58
A	WO 96 09805 A (ZONAGEN INC) 4 April 1996 (1996-04-04) the whole document	1-58

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/21591

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 0368253	A	16-05-1990	US 4946870 A	07-08-1990
			AU 625075 B	02-07-1992
			AU 4449789 A	31-05-1990
			CA 2002404 A	08-05-1990
			JP 2196728 A	03-08-1990
			KR 9402657 B	28-03-1994
			US 5300494 A	05-04-1994
US 4474756	A	02-10-1984	JP 1194517 C	12-03-1984
			JP 57130922 A	13-08-1982
			JP 58023847 B	18-05-1983
			CH 650684 A	15-08-1985
			FR 2499414 A	13-08-1982
			GB 2096146 A, B	13-10-1982
			IT 1189215 B	28-01-1988
			KR 8702162 B	14-12-1987
			US 4814169 A	21-03-1989
WO 9609805	A	04-04-1996	AU 688603 B	12-03-1998
			AU 3683095 A	19-04-1996
			CA 2200550 A	04-04-1996
			EP 0789590 A	20-08-1997
			US 5912000 A	15-06-1999

## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>27779/35932/PCT</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, Item 5 below.	
International application No. <b>PCT/US 99/ 21591</b>	International filing date (day/month/year) <b>16/09/1999</b>	(Earliest) Priority Date (day/month/year) <b>17/09/1998</b>
Applicant <b>ZONAGEN, INC. et al.</b>		

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## 6. The figure of the drawings to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☐ None of the figures.

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/21591

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/00 A61K39/39 //C07K14/59

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 368 253 A (UNION CARBIDE CHEM PLASTIC) 16 May 1990 (1990-05-16) column 2, line 32 - line 47 column 10, line 37	1
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	-/--	

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"&" document member of the same patent family

Date of the actual completion of the international search

2 February 2000

Date of mailing of the international search report

23/02/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Fernandez y Branas, F

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	WO 96 09805 A (ZONAGEN INC) 4 April 1996 (1996-04-04) the whole document	1-58

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

T/US 99/21591

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 9609805 A	04-04-1996	AU 688603 B	12-03-1998
		AU 3683095 A	19-04-1996
		CA 2200550 A	04-04-1996
		EP 0789590 A	20-08-1997
		US 5912000 A	15-06-1999



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US99/21591 <b>(22) International Filing Date:</b> 16 September 1999 (16.09.99) <b>(30) Priority Data:</b> 60/100,766 17 September 1998 (17.09.98) US <b>(71) Applicant (for all designated States except US):</b> ZONAGEN, INC. [US/US]; 2408 Timberloch Place, B-4, The Woodlands, TX 77380 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> HARRIS, Jeffrey [US/US]; 15 Flatstone, The Woodlands, TX 77381 (US). MARTINEZ, Mitzi [US/US], 10731 Fi-Terry Road, Conroe, TX 77303 (US). <b>(74) Agent:</b> UHL, Jill, E.; Marshall, O'Toole, Gerstein, Murray & Borun, 6300 Sears Tower, 233 South Wacker Drive, Chicago, IL 60606 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> HUMAN CHORIONIC GONADOTROPIN VACCINES		
<b>(57) Abstract</b>  A method for the production of the $\beta$ -subunit of human chorionic gonadotropin ( $\beta$ hCG) proteins using recombinant technology, novel DNA sequences encoding such proteins, fragments, thereof, or analogs thereof, and the use of these recombinant proteins combined with adjuvant as a means of interrupting fertility in mammals by the immunological inactivation of the pregnancy hormone hCG.		



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# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/21591

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/00 A61K39/39 //C07K14/59

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 368 253 A (UNION CARBIDE CHEM PLASTIC) 16 May 1990 (1990-05-16) column 2, line 32 - line 47 column 10, line 37	1
X	DIRNHOFER STEPHAN: "The suitability of human chorionic gonadotropin (hCG)-based birth-control vaccines" IMMUNOLOGY TODAY, vol. 15, 1994, pages 469-474, XP002129457 cited in the application the whole document	13-17, 47-51, 58

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☒ Further documents are listed in the continuation of box C.

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JONE W.R. ET AL: "PHASE 1 CLINICAL TRIAL OF A WORLD HEALTH ORGANISATION BIRTH CONTROL VACCINE" THE LANCET, vol. I, no. 8598, 1988, pages 1295-1298, XP002129534 cited in the application the whole document	13-17, 47-51,58
A	US 4 474 756 A (MITSUHASHI MASAKAZU ET AL) 2 October 1984 (1984-10-02) the whole document	1-58
A	WO 96 09805 A (ZONAGEN INC) 4 April 1996 (1996-04-04) the whole document	1-58

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Information on patent family members

International Application No

PCT/US 99/21591

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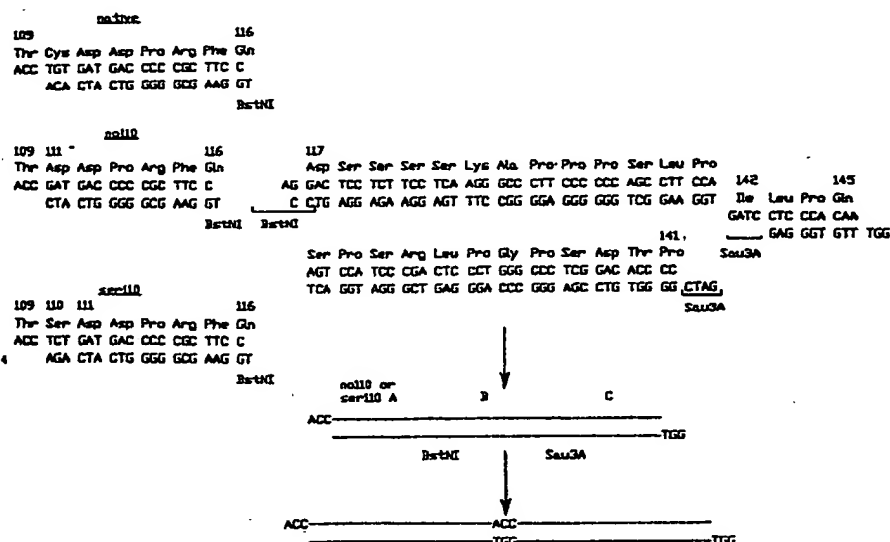
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(21) International Application Number: PCT/US86/01226 (22) International Filing Date: 4 June 1986 (04.06.86) (31) Priority Application Numbers: 741,168 756,847 (32) Priority Dates: 4 June 1985 (04.06.85) 18 July 1985 (18.07.85) (33) Priority Country: US (71) Applicant: BIOTECHNOLOGY RESEARCH PART- NERS, LTD. [US/US]; 2450 Bayshore Frontage Road, Palo Alto, CA 94043 (US). (72) Inventors: TALMADGE, Karen, D. ; FIDDES, John, C. ; 689 Wildwood Lane, Palo Alto, CA 94303 (US).		(74) Agents: MURASHIGE, Kate, H. et al.; Ciotti & Mu- rashige, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025-3471 (US).  (81) Designated States: AT (European patent), AU, BE (Eu- ropean patent), CH (European patent), DE, DE (Eu- ropean patent), DK, FR (European patent), GB, GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (Euro- pean patent).  Published <i>With international search report.</i>

**(54) Title:** AUTOANTIGEN VACCINES

**(57) Abstract**

Autoantigen vaccines are obtained by conferring antigenicity by formation of multimers, of fusion proteins to non-bacterial sequences, or both. The vaccines can be administered using conventional dosage forms for peptide vaccines, or can be supplied in a recombinant vaccinia and the peptides synthesized *in situ*. Particularly useful autoantigens are those derived from hormones controlling reproduction. An easily administered and reversible vaccine for preventing human pregnancy is disclosed. The vaccine comprises a vaccinia virus vector modified to contain the DNA sequence encoding the C-terminal portion (CTP) of the  $\beta$ -chain designed so that the encoded antigen provide a chimera which is capable of expressing the CTP<sub>n</sub> antigen at the cell surface portions thereof, are used as the autoantigen. The derived sequences are also effective autoantigens are also useful.



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AUTOANTIGEN VACCINES

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Technical Field

10           This invention relates to construction and  
design of vaccines which neutralize the effects of  
indigenous proteins. Such vaccines can be made species  
specific or can have cross-species effectiveness. They  
are particularly useful in the control of fertility in  
15 both humans and animals. An important embodiment of  
such vaccines utilizes vaccinia-borne antigens.

Background Art

20           Control of population is a long-standing  
problem of humankind. It has even been suggested that  
the Trojan War was a device of the Zeus to thin out the  
densely populated eastern Mediterranean region (Tuchman,  
B., The March of Folly (1984), p 47; citing Cypria and  
Euripides). More recent efforts have relied on  
25 techniques of slightly more sophistication. While  
methods which require repeated self-administration have  
been moderately successful in some circumstances, many  
individuals, and a few countries as a matter of policy,  
have found it necessary to rely on long-term approaches  
30 such as sterilization or after-the-fact remedies, such  
as abortion. Neither of these seems optimal.

          Control of fertility in the animal population,  
especially among pets, is also a serious problem, at  
least in the United States. Millions of unwanted

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puppies and kittens are destroyed annually due to the failure of the owners of animals adequately to prevent reproduction of their pets. In part, this failure results from the inconvenience and expense of the currently available methods of sterilization, which involve surgical procedures. In addition, there appears to be a psychological reluctance on the part of some pet owners to subject their animals to what they perceive as a drastic procedure. While owners of farm animals are not comparably squeamish, it may still be desirable to offer an alternative method of population and behavior control to castration. For example, it is known that castration of bulls at an early age results in diminution in the quantity of meat, and in a higher fat content. However, alternative means for population and behavior control among pets or domesticated farm animals have, so far, been unavailable.

The general concept of the use of vaccines for the control of fertility in both humans and animals has been the subject of some considerable research. In the case of humans, for example, it has been suggested to immunize females against sperm (Li, T.S., Obstet Gynec (1974) 44:607-623) and also against placental and trophoblastic antigens, which in early work utilized fairly crude extracts (Koren, Z., et al, M J Obstet Gynec (1968) 102:340-346; Beck, J.S., et al, J Path (1970) 125-129), and more recently against purer antigens such as human placental lactogen and human chorionic gonadotropin (Stevens, V.C., et al, Am J Repro Immunol (1981) 1:307-314). See also US Patents 4,384,995; 4,526,716; 4,302,386; and 4,201,770; all to Stevens. Synthetic analogs for human chorionic gonadotropin (hCG) have also been suggested as antigens (Matsuura, S., et al, Endocrin 1 (1979) 101:396-401). A



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recent review of immunological methods has been prepared by Stevens, V.C., in Endocrine Mechanisms in Fertility Regulation, Benagiano, G., and Diczfalussy, E., eds., New York: Raven Press, 1983, pp. 141-162, incorporated  
5 herein by reference. This review includes the suggestion that if properly constructed, vaccines having the carboxy terminal peptide of hCG as antigenic component may be useful in humans. In addition, Thau, R.B., et al have, in several papers, referenced below,  
10 suggested that ovine luteinizing hormone (oLH) may be useful in immunizing human females against pregnancy.

One of the difficulties in administration of antigens against pregnancy is in finding a suitable vehicle which is acceptable for injection into humans.  
15 Ordinary adjuvants, such as Freund's complete adjuvant or Freund's incomplete adjuvant are not acceptable in humans (Stevens, V.C., Serono Symposium No. 45, Academic Press, London & New York, (1982), pp. 131-143). Cost factors in preparing a suitable subunit vaccine have  
20 also been recognized as a problem in preparing anti-fertility vaccines for use in less-developed countries. This is particularly poignant because it is in these areas that overpopulation threatens to become a major detriment to further development.

25 With respect to animals, some studies have been made which would be relevant to generating a peptide- or protein-based vaccine to prevent estrus or to destroy male sexual function in animals. Talwar, G.P., et al (Proc Natl Acad Sci (USA) (1985) 82:1228-1231) prepared  
30 monoclonal antibodies against gonadotropin releasing hormone (GnRH), which antibodies were capable, when injected into female dogs, of immediately suppressing the progression of estrus. Fraser, H.M., et al (J Endocr (1974) 63:399-406) had, a decade earlier,

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demonstrated that atrophy of the primary and secondary sex characteristics of male rats could be induced by immunization with GnRH (then called LHRH) conjugated to bovine serum albumin. The conjugate presumably raised  
5 antibodies to GnRH, which interrupted the normal maintenance of sexual function by neutralizing this hormone. Schanbacher, B.D. (J Androl (1983) 4:233-239) later performed a similar study with similar results using male dogs as subjects. Esbenshade, US Patent  
10 4,556,555 disclosed a process for passive neutering of animals by pre-puberty injection of anti-GnRH antiserum.

However, the development of a vaccine to control population and behavior in household pets and farm animals has, apparently, not been seriously  
15 undertaken. Furthermore, vaccines prepared from proteins or peptide conjugates have certain inherent disadvantages. Preparation of the peptides and formulation of the vaccines are expensive, and administration must be done under controlled conditions  
20 by trained personnel. Also, because these vaccines are prepared by conjugation of the autoantigen to a carrier at the protein, rather than at the genetic level, some denaturation inevitably occurs. It is thus difficult to achieve uniform compositions from batch to batch, and  
25 some compositions have lowered antigenicity, resulting in variable immune responses.

An alternative to protein-based vaccines has been known for many years. Smallpox has been successfully eradicated through the use of vaccines  
30 comprised of vaccinia virus. Vaccine comprised of vaccinia virus has tremendous advantages in terms of cost (approximately two cents per dose) and also in terms of handling, as it can be administered as a scratch under nonsterile field conditions and maintains

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its stability at room temperatur when previously freeze-dried. These prop rties of vaccinia have recently been used to advantage in preparing vaccines against other infectious agents by recombining the genes encoding the desired immunogens against, for example, hepatitis B, herpes, influenza, and rabies, into the vaccinia genome. The recombined vaccinia can then be used in a manner analogous to the original smallpox vaccine, with all the attendant advantages. See, for example, Smith, G.L., and Moss, B., Biotechniques (1984) 306-312; Smith, G.L., Nature (1983) 302:490-495; Panicali, D., et al, Proc Natl Acad Sci (USA) (1983) 80:5364-5368; Paoletti, E., et al, Proc Natl Acad Sci (1984), 81:193-197; Wiktor, T.J., Proc Natl Acad Sci (1984) 81:7194-7198; and Panicali, D., et al, Proc Natl Acad Sci (1982) 79:4927-4931.

The desirable properties of the vaccinia carrier have, however, never been marshalled to administer effective vaccines directed against autoantigens. The appropriate autoantigens for control of fertility depend on the species of the subject, but are generally related to those hormones which regulate the reproductive system. Human chorionic gonadotropin is a particular such antigen which, if neutralized, would be incapable of its necessary role in the maintenance of human pregnancy. Thus, raising neutralizing antibodies against this factor, for example through the vehicle of vaccinia-borne administration would result in effective control of the consequences of conception in humans. The use of chorionic gonadotropin carboxy-terminal peptide is not workable in animals in general, however, because there is no placental production of chorionic gonadotropin in most species except horses. Therefore, for vaccines effective in

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non-human species to achieve sterilization or temporary infertility, alternative autoantigens must be used. DNA sequences encoding a number of hormones known to be significant in controlling the estrus cycle, such as luteinizing hormone (LH) and follicle stimulating hormone (FSH) are usable. However, in a particularly preferred embodiment, the DNA encoding GnRH is effective in generating antibodies which prevent fertility in these animals. As the sexual function of both male and female farm animals and in dogs and cats is controlled primarily by GnRH, either sex may be rendered infertile or sterilized using such vaccines.

#### Disclosure of the Invention

The invention provides vaccines which are low-cost, effective preparations suitable for use in developing countries as well as in the industrialized world for fertility control, both in humans and in animals. The vaccines are also useful, in general, to control any metabolic function which is regulated by an indigenous protein or autoantigen.

The vaccines include those which provide protection against pregnancy in humans for limited time periods and which are reversible so as to permit their use without the negative effect and disincentive of permanent sterilization. Such vaccines are immunogenic forms of human chorionic gonadotropin (hCG), a placental product necessary for maintenance of pregnancy. Other hormones connected with reproductive function, such as GnRH, and certain forms of LH can also be used as the basis for providing antigenic determinants in the human vaccine. Analogous vaccines using autoantigenic forms of, for example, GnRH, FSH, or LH are useful in fertility control in animals. The immunogens can be

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administered as DNA constructs with vaccinia virus, a vehicle which permits low-cost formulation as well as administration under a range of conditions some of which may be less than desirable for delivery of medical services. They can also be administered in more conventional methods such as protein vaccines using, for example, an autoantigenic construction encoding the appropriate region of the appropriate hormone as the antigenic determinant.

10           The invention, however, has broader application. Indigenous proteins in general can be made effective as autoantigens by multimerization of their epitopes or by conjugation to a non-bacterial amino acid sequence in the form of a fusion protein and can be  
15 administered in conventional or vaccinia-borne forms. Thus, any metabolic function regulated by peptides is susceptible to control by appropriate vaccines. The species specificity of such vaccines can be controlled since this depends upon the choice of epitopes. The  
20 vaccines can thus be constructed to be broadly applicable across species lines, or can be limited in their effect to a single species of choice.

          In its broadest aspect, the invention relates to the use of autoantigens as vaccines to control  
25 metabolic function wherein the antibodies to an appropriate indigenous protein are raised in response to a recombinantly created "autoantigenic" form, such as, preferably, a multimer which contains at least 2 repeating units of at least one epitope associated with  
30 the indigenous protein, or as a recombinantly produced fusion protein bearing a suitable epitope. The autoantigenic forms are constructed at the DNA level, using recombinant techniques, and the autoantigenic proteins may either be generated in situ in the subject, if the DNA encoding the autoantigen is properly disposed

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in a vaccinia vehicle, or they may be produced using recombinant techniques and supplied as peptides or proteins.

In one approach, the autoantigen is a multimer, and the DNA encoding an indigenous peptide, such as a hormone, or an epitope-containing portion thereof, is manipulated in vitro to obtain repeating coding sequence for a multimer of this sequence. The proteins which form the basis for the vaccine may also be monomers with respect to an autoantigen epitope if they are disposed in suitable carrier proteins which provide sufficient immunogenicity. The DNA encoding this epitope is, in this case, ligated to the DNA encoding the carrier. Finally, the epitope-containing moiety can be linked to a carrier to create immunogenicity at the protein level.

In one particular aspect, the invention relates to antipregnancy vaccines useful in humans which are based on epitopes of a specific region of the hCG protein - the carboxy terminal peptide (CTP) which represents the end of the "β-hCG" chain. (As will be explained below, hCG contains two non-covalently bound subunits, designated α and β.) These monomers and multimers include CTPs modified by containing no cysteine at position 110 or having cysteine replaced with a serine or other equivalent amino acid residue at position 110. In another embodiment, vaccines against pregnancy in farm animals and pets, and, if properly administered, in humans, are based on the production of immunogenic forms of a fertility controlling autoantigen gonadotropin releasing factor (GnRH) a product of the hypothalamus which controls release of certain hormones from the pituitary. The most preferred forms of GnRH are modifications of the human sequence wherein the N-terminal pyroglutamic acid (presumably formed by

cyclization in serum) is replaced by glutamine and the C-terminal amidated glycine is replaced by glutamine. Still other embodiments utilize immunogenic forms of other fertility controlling autoantigens, specifically, 5 LH and FSH, in both animals and humans.

Also an aspect of the invention are vaccines whose species specificity is regulated by the choice of epitopes in converting portions of human, bovine, and dog LH  $\beta$  chains to autoantigens. Depending on the 10 portions of the peptides chosen, these vaccines are species-specific or species-versatile in their application.

In all cases, the desired autoantigen can if desired be administered in the form of a recombinant 15 vaccinia containing expressible monomers and multimers of the autoantigen. For vectors whose DNA sequence encodes only the autoantigen (for example, the multimers of CTP or GnRH), intracellular expression is expected to result. These antigens will be revealed to the immune 20 system when the infected cells in which they reside are lysed by the cytotoxic immune response mounted against vaccinia. For those embodiments wherein the vaccinia genome is recombined with a carrier protein encoding sequence (such as influenza hemagglutinin (HA) encoding 25 DNA) which is interrupted by the DNA sequences encoding the desired autoantigen, the secondary carrier functions to transport the autoantigen monomers or multimers to the surface of infected cells. This creates more effective exposure of the autoantigen to the subject, 30 and enhances the immune response. In one embodiment, using HA, for example, the desired autoantigen genes are cloned into the A or B antigenic regions of the influenza HA; in a second embodiment, they are cloned between the HA signal sequence and membrane anchor

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portions. Both of these approaches result in presentation of the antigen at the surface of the cell. Other constructions result in the secretion of the expressed protein. For instance, the desired autoantigen DNAs can be cloned into HA encoding DNA so that the resulting proteins are linked operably to the signal sequence, but with the membrane anchor portion of the HA eliminated. Alternatively, other signal sequences, such as, for example, those associated with bacterial alkaline phosphatase (BAP) or renin can be used to effect secretion of the the desired autoantigen immunogens, or the signal sequence associated with the native antigenic protein may be employed.

In other aspects, the invention relates to methods of preventing pregnancy or controlling other metabolic functions by administration of the foregoing vaccines and to methods of preparation of such vaccines, as well as to intermediate vectors and DNA sequences useful in their preparation.

In still another aspect, the invention relates to antibodies produced in response to these vaccines. These antibodies are particularly useful in assessing the levels of the hormones themselves through immunoassay. Accordingly also included within the invention are labeled and unlabelled forms of the these antibodies, and these antibodies both in soluble form and bound to a solid support. Spleens obtained from animals immunized with the vaccines of the invention can also be used as a source of cells for immortalization to obtain corresponding monoclonal preparations.

#### Brief Description of the Drawings

Figure 1 shows the DNA sequences useful in preparing the gene encoding modified and unmodified



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$\beta$ -hCG CTP monomers and multimers representing amino acids 109-145 of  $\beta$ -hCG.

Figure 2 shows the amino acid sequence for GnRH in several species and the DNA sequences useful in preparing the genes encoding modified human and chickenII GnRH monomers and multimers.

Figure 3a shows the cDNA sequence of and method of constructing appropriate multimers of a portion of the  $\beta$  peptide of bovine LH.

Figure 3b shows the cDNA sequence of and method of constructing appropriate multimers of a portion of the  $\beta$  peptide of dog LH.

Figure 4 shows the cDNA sequence of and method of constructing appropriate multimers of a portion of the  $\beta$  peptide of human LH.

Figure 5 shows modification of pKK223-3 to obtain the host vector pVAL and of pKT19 and pKT41 to obtain the host vector pVA2.

Figure 6 shows the construction of derivative vectors pHA1 and pHA2 containing an HA-encoding DNA.

Figure 7 shows a map of a typical hemagglutinin encoding gene with useful restriction sites for its use as a carrier.

Figure 8 shows the modification of the HA gene for insertion of autoantigen multimers in the HA antigenic regions.

Figure 9 shows the construction of intermediate vectors carrying an HA gene modified by insertion of autoantigen between its signal sequence and membrane anchor.

Figures 10a, 10b, and 10c show the construction of intermediate vectors containing autoantigen inserts into an HA secondary carrier which lacks membrane anchor codons.

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Figures 11 and 12 show the construction of vectors designed to effect autoantigen secretion using the signal sequences of BAP and renin, respectively.

Figure 13 shows the construction of pGS20 and a diagram of its use in effecting integration of a desired sequence into the vaccinia virus TK gene.

Figure 14 shows the results of a gel analyzing the proteins produced by cells transfected with expression vectors for the autoantigenic CTPs of the invention.

Figure 15a shows a comparison of human, rat, bovine, and dog  $\beta$  LH amino acid sequences. Figure 15b shows the location of peptides useful in species-specific vaccines, and the sequences of oligonucleotides encoding them.

#### Modes of Carrying Out the Invention

The invention, in one aspect, relates to methods and constructions which utilize the vaccinia system to introduce and express genes for autoantigens in vertebrate hosts. Heretofore, vaccinia has been used as a vehicle for DNA sequences which express proteins that are already immunogenic in the target host, and have not been employed to obtain expression of autoantigens that are altered to render them immunogenic. A particularly suitable "alteration" includes use of multimeric genes to produce multimeric antigens, so that the joined regions form the foreign epitopes. Immunogenicity is also provided when monomeric as well as multimeric genes inserted into DNA regions encoding known antigenic sites, such as those which reside in influenza hemagglutinin. The autoantigen will then be presented in a part of the protein known to be accessible to antibodies, and the

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other hemagglutinin antigenic sites as well as the joining regions to the antigen region sequence represent the foreign epitopes. These and other constructions employing monomeric and multimeric genes in foreign proteins use these foreign proteins to carry and dispose the resulting chimeras to the infected cell surface or to secrete them from the infected cell. This transport makes the autoantigen-bearing chimeras accessible to the immune system.

10           As the proteins which result from in situ expression of these recombinant constructs are immunogens, the immunogens could also be produced outside the subject using recombinant techniques and administered as conventional protein vaccines. This  
15 recombinant expression includes the use of conventional procaryotic and eucaryotic expression systems and hosts, as well as transient expression from a recombinant intermediate shuttle vector using standard transformation into vaccinia-susceptible cells and  
20 infection with vaccinia virus.

          As the vaccines are effective in producing antibodies which are immunospecific for the hormones involved in reproduction or for other indigenous proteins, these antibodies can, of course, be purified  
25 and used in immunoassays for the determination of the antigen.

#### A. Conferring Immunogenicity on Indigenous Proteins

          At the heart of the invention is the use of  
30 recombinant techniques to create forms of indigenous proteins which are immunogenic, and which therefore behave as autoantigens.

          These "autoantigens" are portions of or complete sequences of proteins normally made in the

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h st, and which proteins are thus immunologically privileged, unless they are made immunogenic. This immunogenicity can be obtained by linking the gene for an autoantigen which represents all or part of the immunologically privileged protein to a gene for an additional sequence, either a duplicate of the autoantigen or another secondary carrier protein, or both, so that the junction region between the autoantigen and its adjacent amino acids is clearly foreign to the host organism and is capable of conferring immunogenicity on the entire protein. The chimeric proteins produced by the inserts into vaccinia virus exhibit this property when produced in situ, or the same coding sequences can be produced using recombinant expression techniques in vitro and then used as conventional vaccines. In some cases, especially where the autoantigen is only a portion of an immunologically privileged protein, the autoantigen alone may be immunogenic.

In summary, the invention concerns recombinantly produced immunogenic forms of indigenous proteins. The immunogenic form obtained by constructing the gene encoding and producing either: a multimer which consists essentially of at least 2 repeating units of at least one epitope of the indigenous protein, or at least one epitope of the indigenous protein conjugated to a non-bacterial polypeptide, or a multimer which consists essentially of at least 2 repeating units of at least one epitope of the indigenous protein conjugated to an additional polypeptide sequence.

According to one embodiment of the invention, multimers of the desired autoantigen are created at the DNA level. The junction regions of the multimeric proteins constitute the foreign sequences which confer

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immunogenicity on the resulting polymeric protein. This approach has the advantage of generating a relatively large immunogen with few irrelevant epitopes, and with a high concentration of the desired determinant, as compared, for example, with the more traditional conjugation of the desired hapten with a carrier protein. Alternative ways to create epitope polymers using polymerization agents which directly act upon the peptide, such as glutaraldehyde, result in denatured forms of the epitopes, and are therefore sometimes of marginal efficiency in producing the desired constructs. Using the techniques of the invention, the protein is "polymerized" at the DNA level, resulting in a recombinant polymer which more closely resembles the native peptide form. Recombinant multimers have been constructed from proinsulin (Shen, S., Proc Natl Acad Sci (USA) (1984) 81:4627-4631), and the multimers expressed in E. coli. These multimers were used to stabilize the recombinant protein, and were subsequently depolymerized to regenerate the monomeric form, which was the desired product. Recombinant multimers of the "CS" protein of the human malaria parasite have also been constructed (Young, J.F., et al, Science (1985) 228:958-962). These are peptides encoded by monomers, dimers and trimers of a XhoI fragment which therefore contain 16, 32, or 48 tandem copies of a repeated tetrapeptide sequence. The native protein contains 37 repeats of this tetrapeptide interspersed with 4 identical (to each other) tetrapeptides. The peptides are found to be immunogenic in mice.

The immunogenicity of the protein may also be enhanced by inserting it into the antigenic regions of a protein derived from an infectious material such as a

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viral surface prot in. or to a carrier which functions simply to increase the size of the peptide.

The "carrier" is a peptide sequence resulting in the production of a fusion protein between at least one epitope of the desired autoantigen and a non-bacterially derived sequence capable of conferring immunogenicity on the epitope which provides the hapten for the immunogen. Again, the junction region represents unfamiliar sequence to the recipient, and the additional peptide confers sufficient size, if needed, to raise antibodies against this determinant. The peptide sequences of the influenza hemagglutinin protein are particularly useful in providing the needed additional amino acid sequence.

A diagram of a typical hemagglutinin (HA) gene is shown in Figure 7. The particular map shown is of the antigenic variant A/Japan/305/57 (H2) as disclosed in Gething, M.J., et al, Nature (1980) 287:301. As indicated in the figure, the gene encodes a signal sequence, a peptide containing approximately 550 amino acids followed by a membrane anchor sequence. The gene is framed by *BalI*, *NdeI*, and *PstI* restriction sites and contains several convenient restriction sites in the body of the amino acid sequence. The epitopes, either as monomers or as multimers, may be ligated into the antigenic regions shown as A and B by providing blunt end restriction sites between codons in these regions using site specific mutagenesis. In addition, the naturally occurring restriction sites may be used to result in proteins extended by the remaining portions of the HA carrier.

If the fused gene is expressed in culture outside the host to provide the fused protein, the HA sequences serve merely to provide the desired

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immunogenicity on the construct. Of course, retaining the signal sequence and deleting the membrane anchor in the cultured cells will result in the ability to recover the desired protein from the culture medium. If  
5 expressed in situ in the vaccinated host, the HA sequences serve functions more directly related to the presentation of the antigenic determinant to the host.

Of course, multimer encoding sequences may be ligated to DNA encoding carrier in a combination of the  
10 foregoing methods.

An additional advantage may be obtained by the use of vaccinia as carrier for any autoantigenic gene constructed as described above in that the levels of immunogenicity may be adjusted through the nature of the  
15 protein encoded. As stated above, if the autoantigen is inserted directly into vaccinia, the encoded autoantigenic protein will be produced as an intracellular protein with limited exposure to the immune system. Insertion into an antigenic region of a  
20 viral protein will result in display of the antigen on the cell surface; similar enhancement may be obtained by using additional secondary carrier sequences which result in secretion from the infected cell or disposition on the cell surface.

25 The autoantigens are constructs of peptides which are involved in metabolic control of the subject host, especially hormonal control, and, in particular, fertility control.

The invention is illustrated below with respect  
30 to specific useful embodiments. In one of these, coding sequences derived from  $\beta$ -hCG are made immunogenic and are thus useful in creating vaccines which prevent pregnancy in humans. In two other specific illustrations, GnRH and LH of known sequence are made in

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a form to confer infertility on a variety of animals. Human GnRH is illustrated for use in animals in general, including dogs and cats. However, also preferred are other GnRH peptides of known sequence, such as the GnRH peptides shown for salmon and chicken in Figure 2. It should be noted that porcine and bovine sequences are identical with each other and with the human sequence. Presumably, in view of the high degree of sequence homology with species as distant as salmon, the GnRH native to dogs or cats or to other farm animals will at least have immunological cross-reactivity with the human and or chicken forms.

Both bovine, dog, and human LH  $\beta$  peptides are illustrated. These may have considerable cross-species reactivity, especially if regions of homogeneity are included. However, as illustrated below, species specific vaccines may also be constructed by recombinantly generating peptides or multimers of peptides which represent non-homologous regions.

In addition, other autoantigens which are fertility controlling autoantigens can also be used and include, for example, those derived from ovum proteins such as zona pellucida peptide; sperm antigens such as hyaluronidase and acrosin; the sperm-specific isozyme of lactose dehydrogenase, and the additional pituitary hormone follicle stimulating hormone (FSH).

In particular, certain approaches to fertility control are considered particularly desirable. For humans, vaccines which succeed in immunizing the subject based on the CTP portion of  $\beta$  HCG are particularly preferred, but it is also effective to utilize the entire  $\beta$  HCG subunit or other portion thereof, or the ovine  $\beta$  LH subunit or portion thereof to prevent pregnancy in humans. Thau, R.B., et al. Fertil Steril (1979) 31:200-204; ibid (1980) 33:317-320; ibid, Endocrinol



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(1983) 112:277-283; and Yamamoto, Y., et al, J Reprod Fertil (1982) 4:295-311; J Reprod Immunol (1983) 5:195-202 have shown that ovine  $\beta$  LH is effective in preventing pregnancies in Rhesus monkeys. In addition, vaccines which confer immunity against FSH (Mougдал, N.R., Arch Androl (1981) 7:117-125; Mougдал, N.R., et al, Am J Reprod Immunol (1985) 8:120-124) and GnRH are administerable to decrease fertility in human males, although the administration of vaccines which raise antibodies against GnRH must be accompanied by administration of testosterone or other steroids which prevent loss of libido.

In animals, vaccines which are specific in raising antibodies against GnRH are preferred. Also particularly useful are vaccines which are species specific -- i.e., which are capable of preventing pregnancy or inducing sterility in only the species to which they are administered. These vaccines utilize epitopes which reside in regions of the hormones controlling reproduction which are non-homologous between species, particularly between humans and species to which humans must administer the vaccines. This may be especially desirable in constructing a vaccine useful in animals, as there would then be little danger of affecting the humans responsible for its administration. The illustration below sets forth an exemplary procedure whereby a region of low homology is chosen as the antigenic determinant for a vaccine.

In a converse application, the gene encoding inhibin, a hormone which affects fertility by lowering the ability of the pituitary to secrete FSH can be used; here the antibodies would increase the fertility of the host. See Olson, P.W., et al, Symposium on Endocrinology, Vet Clin NA: Small Animal Practice

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(1984) 14:927-926. It also may be desirable to immunize the host against these and other hormones in order to regulate overproduction of these hormones and the resulting metabolic aberrations. In general, the autoantigens with which the invention is most concerned are "fertility controlling"--i.e., hormones which are involved in the physiological control of sex characteristics including behavior and fecundity.

In another context, it has been disclosed by Reddy, V.B., et al, Proc Natl Acad Sci USA (1985) 82:3644-3648, PCT Applications Publication Nos WO85/01958 and WO85/01959, that simultaneous enhanced expression of genes encoding both  $\alpha$  and  $\beta$  subunits of the fertility hormones is achievable using an autonomously replicating vector in monkey cells. No production of the  $\beta$  chain of hCG was, however, observed if sequences encoding the  $\alpha$  chain were omitted from the vector.

There are a large number of additional autoantigens to which it is desirable to generate neutralizing antibodies. A partial list of such autoantigens would include those corresponding to immunoglobulins generated by the victims of autoimmune diseases which are specific to the host's own tissues (where the neutralizing antibodies would suppress the autoimmunity) and those corresponding to the T24 bladder carcinoma growth factor (Taparowsky, E., et al, Nature (1982) 300:762), a protein generated by a human oncogene which causes the transformation of bladder cells (where the antibodies would retard the development of malignancy).

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## B. CTP Immunogens

Th most preferred embodiment of vaccines against human pregnancy are those wherein the autoantigenic peptides are derived from the carboxy terminal peptide (CTP) of the  $\beta$  chain of human chorionic gonadotropin ( $\beta$ -hCG). Use of CTP-related peptides has several advantages:

First, while the CTP is derived from a human protein which is associated with a human hormone similar to certain others specified below, the CTP region is unique to hCG, thus minimizing the likelihood of unwanted interference with other essential hormones by antibodies raised against CTP. hCG itself is a glycoprotein of molecular weight 38 kd, of which 30% represents sugar. The protein has two dissimilar subunits,  $\alpha$  and  $\beta$ -hCG, which are coupled by noncovalent linkage. The  $\alpha$  subunits of hCG and of three other hormones--luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH) are identical in humans. hCG is secreted by the placenta; LH, FSH, and TSH are secreted by the pituitary. The  $\beta$  subunits of all four of these hormones show differences between them, but do retain some amino acid sequence homologies. The homologies are greatest between hCG and hLH  $\beta$  subunits; however, hCG contains an additional thirty amino acids extended beyond the 115 amino-acid residues in the  $\beta$  subunit of hLH. In general, positions 111-145 of  $\beta$ -hCG show no counterpart in hLH. Indeed, the peptide represented by positions 109-145 of hCG, conjugated to a carrier protein such as bovine gamma globulin or diphtheria toxoid, has been tested as an antifertility agent in baboons (Stevens, Serono Symposium (supra)).

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A second advantage of utilizing the CTP region of  $\beta$ -hCG is that the hormone which it represents, as a placenta-derived hormone, is not generated directly by the host. Thus, antibodies raised against hCG which are not cross-reactive with FSH, LH, or TSH do not interfere with a hormone which might regulate the metabolism of the immunized subject. The role of hCG is presumed to be stimulation of steroid (progesterone) production by the corpus luteum following conception, until steroid production by the placenta itself can be established. Production of hCG by the placenta apparently begins within six days of conception, and is essential for the maintenance of pregnancy. If sufficient levels of hCG are not synthesized by eleven days, the corpus luteum regresses and normal menstruation occurs; the effect of this failure would presumably be mimicked by neutralizing antibodies. Immunization against hCG thus represents immunization against a non-maternal (although immunologically privileged) hormone which is apparently designed specifically for the maintenance of pregnancy and is presumptively not secreted for any other additional purpose.

A third advantage of using a partial sequence of hCG is that a vaccine using CTP as the immunogen provides sterility that is apparently reversible, as judged by preliminary tests on baboons. (Stevens, supra). This reversibility is thought to be due to the direction of T-cell memory not against native hCG but against the hCG/carrier protein joining regions. Therefore, production of the native hormone in intermittent pregnancies (which are presumably then terminated by the antibodies) is not likely to boost the anti-hCG response, and this has been shown in baboons (Stevens, supra). The ability to construct the CTP

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portion as an unfamiliar protein may indeed account, in the previously disclosed constructions, for the immugenicity of what might otherwise be considered an indigenous protein.

5 (Indeed, this feature is not limited to CTP but is true of autoantigens in general. The subjects are not boosted by their own hormone or endogenous peptide production.)

10 While preliminary experiments have been performed by Stevens (supra) indicating that the CTP region has possibilities as an antifertility vaccine, it has been apparent that a satisfactory formulation for administration of the immunogenic sequence has not been found. Vaccinia offers such a possibility by providing  
15 a means for producing large quantities of immunogen in situ, as well as constituting a vehicle which has the benefit of collective experience through smallpox vaccinations and, more recently, through experimental immunizations against other diseases, as outlined  
20 above.

This is not to say that alternate constructions are excluded. Fusion proteins containing the CTP monomer or multimer may be made using conventional recombinant techniques, and used in a manner common to  
25 traditional subunit vaccines.

#### C. GnRH<sub>n</sub> Immunogens

Gonadotropin releasing hormone (GnRH) is a decapeptide secreted by the hypothalamus and controls  
30 the release of both luteinizing hormone (LH) and follicle stimulating hormone (FSH) in vertebrates (Fink, G., British Medical Bulletin (1979) 35:155-160). These hormones, in turn, control the levels of steroid hormones such as estradiol and progesterone. The amino

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acid sequence of the decapeptide GnRH has been determined for several species, as shown in Figure 2, and the cDNA for human GnRH has been cloned, so that its sequence is established beyond doubt (Seeburg, P.H., et al., Nature (1984) 311:666-668). (GnRH was formerly designated LHRH and is so named in the Seeburg paper.) As the peptide sequences for the GnRH from salmon and two such factors from chicken are known, DNA constructs encoding them are also easily prepared.

Both LH and FSH are significant in regulating the estrus cycles of dogs and cats, although different patterns are obtained. In the dog, luteinizing hormone peaks sharply at the onset of estrus and falls off as the period of estrus proceeds and is dissipated. FSH also reaches a peak at this time, although the peak is slightly retarded from that of LH and the patterns are not precisely the same. The levels of estradiol (controlled by FSH) and progesterone (controlled by LH) also cycle. Therefore, the estrus cycle is destroyed by preventing secretion of LH and FSH from the pituitary. This can be accomplished by suppression of GnRH activity through immunoreaction.

In cats, the orchestration is different, but the players are the same. The surge of LH essential for ovulation is not released except upon sexual stimulation. However, the levels of LH and FSH that control sexual behavior and receptivity do cycle and are controlled by GnRH as in other vertebrates (Johnson, L.M. et al., Endocrinol (1981) 109:240-246). Thus, prevention of LH and FSH secretion by suppressing the activity of GnRH will result in temporary sterility.

In addition, earlier studies have shown that antibodies to GnRH suppress sexual function in males (Fraser, et al. and Schanbacher, et al. supra). Thus,

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vaccines relying on generation of GnRH as antigen are effective in male subjects as well.

An advantage of employing GnRH is the cross species effectiveness of a single vaccine. The amino acid sequence of GnRH is highly conserved among species. As shown in Figure 2, human, sheep, rat, pig, and cow GnRH are completely homologous; the most highly differing peptide is one of two derived from chickens which shows only three amino acid residue differences. All of these have biological activity in rats.

The constructs used in the present invention are derived from the human sequence but are modified by substituting the glutamine residue at the N-terminal position in place of the pyroglutamic acid. This substitution has the effect of retaining the glutamic acid structure but preserving the uncharged structure of the pyroglutamate. The resulting peptide thus does not require cyclization of the glutamic acid residue, and can be conveniently produced in the cytoplasm of the infected cell without concern for the presence of conditions to effect this processing, such as are ordinarily found in serum. The multimer as obtained from these constructs will have a C-terminal glutamine which mimics the amidation of the naturally occurring decapeptide, also obviating the need for subsequent processing.

With respect to obtaining suitable vaccines using GnRH as a base, the considerations discussed above with respect to CTP apply. Administration may be as a vaccinia virus construct or as a conventional subunit vaccine.

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#### D. Luteinizing Hormone and Species Specificity

Vaccines are also illustrated below which are derived from human, bovine, and dog LH  $\beta$  chain sequences. As outlined above, LH is involved in maintenance of the corpus luteum, and although regulation of its level varies from species to species, it is essential for fertility in all. Therefore, antibodies capable of neutralizing LH are effective antifertility agents.

Figure 15a shows a comparison of the amino acid sequences of human, rat, bovine, and dog LH. Many regions are highly homologous, but the boxes designate regions where the differences from human sequence occur. Vaccines based on raising antibodies to LH can thus be made species-specific (or at least, ineffective in humans) by selecting these regions of low homology to obtain the autoantigen. In general, species specificity is obtained by selecting non-homologous regions, or vaccines can be made effective across species lines by using homologous regions. Figure 15b suggests peptides of interest for cow-specific and dog-specific vaccines.

The non-homologous peptides indicated are sufficiently short that genes encoding them can easily be made using the currently available chemical synthesis methods. Thus, the monomeric and multimeric units analogous to those of CTP and GnRH described above can be synthesized and manipulated in similar ways, either individually, or as cocktails which include two or three of the desired peptides. However, DNA capable of encoding major portions of either the bovine or human sequences can most conveniently be obtained by recovering a cDNA clone from libraries constructed using bovine or human pituitary mRNA. Complete genomic coding sequences for bovine LH (Virgin, J.B., et al, J Biol



Chem (1985) 260:7072-7077) and f r human LH (Talmadge, K., et al, Nature (1984) 307:37-40) are kn wn, and because of high sequence homology, the  $\beta$ -hCG cDNA released as a HindIII fragment from the pBR322 cloning  
5 vector described in Fiddes, J.C., et al, Nature (1980) 286:684-687 can be used as probe. The nucleotide sequence encoding the  $\beta$  chain of rat LH has also been disclosed (Chin, W.W., et al, Proc Natl Acad Sci (USA) (1983) 80:4649-4653). The cDNA sequences encoding cat  
10 or dog  $\beta$ LH can be retrieved from  $\lambda$ gt10 libraries prepared from pituitary mRNAs of the respective species.

Significant portions of the bovine, dog, and human LH sequences can be excised for the construction of autoantigenic forms as described in more detail in  
15 the illustration set forth below. For the bovine cDNA, naturally occurring HaeIII sites at amino acids 22 and 85 provide a convenient fragment; for the human cDNA, a MaeIII site at codons 111-112 can be employed along with an artificially introduced MaeIII site at codons 1-2. In  
20 all cases, these constructions are merely for the purpose of example, and constructions can be made using any major portion of the peptides. The manipulations to effect autoantigenicity - multimerization of the DNA, ligation into a carrier protein, production of a fusion  
25 protein, and so forth; and the means of administration - as a vaccinia borne expression system or as a protein or mixture of proteins produced by recombinant means, are similar to those described in connection with GnRH and CTP.

30 Antibodies raised against the immunogenic proteins of the invention, whether these proteins are produced for vaccine formulation or in situ, are useful in diagnosis and, if correctly prepared, in passive treatment. Conventional processes known in the art are

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used to prepare these antibodies. Both the antibodies themselves and their immunospecific fragments, such as Fab fragments are thus useful.

5 E. Definitions

As used herein, "carboxy terminal peptide" or "CTP" refers to a region of  $\beta$ -hCG which has little in common with the three similar hormones FSH, TSH, and LH.  $\beta$ -hCG is a 145 amino acid peptide of known sequence. (Fiddes, J.C., et al, Nature (1980) 286:684-687). The approximately 30 amino acids representing positions 109-145 represents a unique determinant among this group. As used herein, CTP refers to a peptide having an amino acid sequence substantially similar to that represented by amino acids 109-145 of the native  $\beta$ -hCG. However, it is understood that minor modifications of the amino acid sequence may be made without affecting the immunological characteristics of the resulting peptide. Amino acid additions, deletions, and alterations can be made so long as they do not negatively affect the ability of the resulting peptide to elicit neutralizing antibodies against hCG itself. Such modifications include, but are not limited to, deletion of the cysteine residue at position 110 or replacement of the cysteine residue by serine or another amino acid which does not impair the immunogenicity of the resultant modified form, such as, for example, alanine, glycine, or valine. Deletion of this cysteine residue provides an improved form of the protein as the fortuitous formation of unwanted disulfides is thus prevented.

Also used in the vaccines of the invention are "multimers" of CTP which comprise repeated sequences of the particular CTP peptide employed. These multimers

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may consist of multiples of 2-10 of the CTP peptide and are head-to-tail ligations of the monomer form. Thus the designation CTP<sub>n</sub>, where n is 2-10, designates embodiments which contain 2-10 of the approximately 30-mers corresponding to CTP.

As used herein, "gonadotropin releasing factor" or "hormone" or "GnRH" refers to the hypothalamus product which is generally believed to effect the release of FSH and LH from the pituitary, as well as to perform other regulatory functions relating to sexual activity, and which is typified by the amino acid sequences determined for various vertebrate species as shown in Figure 2. Where the context clearly so requires, GnRH also refers to the appropriate DNA sequence which encodes the referenced peptide. As used herein, GnRH refers to a peptide having an amino acid sequence substantially similar to that represented in Figure 2; however, it is understood that minor modifications of the amino acid sequence may be made without affecting the immunological characteristics of the resulting peptide, and the definition includes these resulting peptides. In the context of the present invention, amino acid additions, deletions, and alterations can be made so long as they do not negatively affect the ability of the resulting peptide to elicit neutralizing antibodies against GnRH itself. Such modifications include, but are not limited to, replacing the pyroglutamic acid residue at position 1 of the human sequence by glutamine, and addition of a glutamine residue at the C-terminus of the monomer or multimer. Such alterations do not impair the immunogenicity of the resultant modified form, and are specifically included within the definition. They, in fact, represent a preferred embodiment. It is also

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clear from Figure 2 that the amino acid residues in positions 7 and 8 are the least conserved. Therefore altered forms of GnRH with substitutions in these positions are effective.

5 Also used in the vaccines of the invention are "multimers" of GnRH which comprise repeated sequences of the particular GnRH peptide employed. These multimers may consist of multiples of 2-10 of the GnRH peptide and are head-to-tail ligations of the monomer form. Thus  
10 GnRH<sub>n</sub>, where n is 2-10, designates embodiments which contain 2-10 of the decamer corresponding to GnRH. The multimers may contain an additional C-terminal amino acid, as is the case in the illustration below.

As used herein, "human LH" or hLH, "bovine LH"  
15 or bLH, and "dog LH" or dLH refer to the respective human and bovine hormones which are responsible for the maintenance of the corpus luteum. In general, the bLH, dLH, and hLH antigens used in the invention have the amino acid sequences shown in Figures 3a, 3b, and 4, or  
20 significant portions thereof. Where the context clearly so requires, b-, d-, or hLH also refers to the appropriate DNA sequences which encode the referenced peptides. As used herein, bLH refers to a peptide having an amino acid sequence or portion substantially  
25 similar to that represented in Figure 3a (dLH to that of Figure 3b; hLH to that of Figure 4); however, it is understood that minor modifications of the amino acid sequence may be made without affecting the immunological characteristics of the resulting peptide, and the  
30 definition includes these resulting peptides. In the context of the present invention, amino acid additions, deletions, and alterations can be made so long as they do not negatively affect the ability of the resulting

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p ptid to licit neutralizing antib dies against the appropriate LH itself.

Also used in the vaccines of the invention are "multimers" of the bovine, dog, and human LH which  
5 comprise repeated sequences of the particular LH peptide employed. These multimers may consist of multiples of a significant portion of the appropriate LH sequence, such as, for example, the amino acids represented by residues 22-85 of the bovine LH 43-115 of dog LH, and 2-111 of  
10 the human LH, and are head-to-tail ligations of the monomer form. Multiples of 2-10 monomers are used. Thus  $bLH_n$ , where n is 2-10, designates embodiments which contain 2-10 of the peptide representing residues 22-85 of the bLH sequence, or its functional equivalent;  
15  $dLH_n$  and  $hLH_n$  designate the corresponding multimers of the sequence of amino acids 43-115 of dog LH and 2-111 of hLH, respectively or their functional equivalents.

Generically, "Hormone<sub>n</sub>" refers to a monomer  
20 (if n is 1) or multimer of the appropriate portion of any particular hormone.

"Autoantigen" refers to a protein or portion thereof which is normally present in the targeted host, at least intermittently, and which normally does not  
25 elicit an immune response from the host. Such autoantigens may ordinarily be made immunogenic only by using them as a hapten through conjugation to a suitable carrier. The carrier may simply be an extension of the amino acid chain; indeed, the extension may simply be  
30 reproduction of the autoantigen in multiple units. The immunogenicity presumably results from the unfamiliar junction regions in this case. Similarly, immunogenicity results from the junction regions when the aut antigen is linked t sequences which serve a

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secondary function, such as providing antigenic epitopes themselves, or serving carrier functions. On occasion, a portion of an immunologically privileged protein may, itself be immunogenic. The term "autoantigen" is used both to refer to the unaltered protein desired to be made immunogenic and to the autoantigen of interest in the immunogenic construct.

"Non-bacterial carrier" refers to a carrier protein which confers immunogenicity on an epitope of the indigenous protein by virtue of enhancing the size of the peptide and/or providing junction regions which contain amino acid sequences foreign to the host. The additional sequence may be any protein of non-bacterial origin, may include viral coat or core proteins, secreted yeast proteins, such as invertase, which have no homologous counterparts in mammals, and membrane bound and secreted proteins from protozoa, such as malaria. Particularly useful carrier proteins are those derived from influenza hemagglutinin. As used herein, "derived from" means that the amino acid sequence is the same as that for a portion containing at least 10 amino acids of the sequence of the source protein, and, of course, is not meant to mean physically obtained from that protein per se. Useful sequences derived from the hemagglutinin protein include portions of the antigenic regions and of the membrane anchor region of this protein.

"Secondary carrier" refers to a protein which is capable of transporting the autoantigen such as CTP or LH or its multimer to the surface of a cell infected with vaccinia virus containing the DNA encoding both the secondary carrier and the CTP or LH or its multimer, or of effecting the secretion of CTP or LH or its multimer from the infected cell. In this regard, the vaccinia

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virus per se is generally referred to herein as a "carrier" for foreign sequences included in its genome. Thus foreign DNAs additional to the DNA encoding autoantigen, such as CTP or LH or its multimer, may  
5 encode proteins which are secondary carriers for the immunogen. As described below, the influenza hemagglutinin protein is representative of secondary carriers useful in this regard. However, other proteins which are known to be transported to the surface of  
10 infected cells may also be used. Such proteins include the neuraminidase peptide of influenza, rabies glycoprotein, and herpes simplex virus (HSV) glycoprotein D. Signal sequences operable in infected cells may be derived from a variety of sources including  
15 preprorenin, preproinsulin, preprorennin, and bacterial alkaline phosphatase. An alternate method of secreting antigen utilizes the pre-S gene of hepatitis. The gene encoding the desired antigen is cloned into the pre-S gene, resulting in secretion of the chimeric protein,  
20 which naturally aggregates to form an empty hepatitis virus coat (Valenzuela, et al, Vaccines 85: Molecular and Chemical Basis of Resistance to Parasitic, Bacterial, and Viral Diseases, New York, Cold Spring Harbor Press, 1985, pp. 285-290).

25 "Operably linked" refers to juxtaposition so that the functionality of the elements is preserved. Thus, for example, promoters operably linked to coding sequences permit their expressions in suitable hosts. Signal sequences operably linked to other peptides (or,  
30 at the DNA level, signal sequence encoding DNA operably linked to peptide encoding DNAs) permit the secretion of the subject peptide from the host cell.

It should be understood that "derived from", when referring to a DNA sequence or amino acid sequence,

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indicates a correspondence in composition to the referenced material, and not necessarily actual physical derivation therefrom. For example, a DNA encoding a signal sequence which is derived from influenza hemagglutinin refers to a DNA constructed so as to encode a peptide sequence substantially similar to that which is found in influenza HA, and that sequence may be constructed by, for example, obtaining cDNA from the HA protein, by synthetic methods using automated oligonucleotide synthesis, or in any other manner designed to recreate a coding sequence for the desired peptide.

#### F. Preparation of Recombinant Vaccinia

The parameters for effecting the integration of foreign DNA into vaccinia are known in the art and are currently in a position for practical utilization. Briefly, the desired immunogen is transferred into a nonessential portion of the vaccinia genome by coinfecting host cells with both native vaccinia and a carrier plasmid which contains the foreign gene sandwiched between sequences homologous with the selected nonessential portion of the vaccinia genome. In addition, the foreign gene is provided with a vaccinia promoter, which will permit its expression under the influence of the vaccinia transcription and translation systems. A general purpose vector capable of housing the foreign gene has been disclosed by Moss, B., et al (Proc Natl Acad Sci (USA) (1983) 80:7155-7159). A diagram of this vector, pGS20, and its manner of use to obtain a recombinant vaccinia virus containing a desired foreign gene is shown in Figure 13.

pGS20 has a vector fragment derived from pBR328, a vector compatible with E. coli which contains



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the E. coli origin of replication and the ampicillin resistance gene. The vector contains the promoter from the 7.5 k gene of vaccinia, excised as a 275 bp HincII/RsaI fragment (Venkatsen, et al, Cell (1981) 5 25:805-813), which is translocated into the EcoRI site of the vaccinia thymidine kinase (TK) gene. The TK gene is obtained as a HindIII/BglII fragment from the HindIII J fragment of vaccinia. Other restriction site modifications have been made for convenience, as shown 10 in Figure 13, and there are BamHI and SmaI restriction sites immediately downstream of the promoter to permit foreign gene cloning.

Thus, pGS20 is constructed as follows: pBR328 is digested with EcoRI, blunted, and religated. The 15 resulting vector is then digested with HindIII and ligated to the HindIII J fragment of vaccinia. The resulting plasmid is digested with BamHI and BglII and religated, thus eliminating the non-TK sequences, along with some unnecessary vector sequences from the insert, 20 as well as eliminating the BamHI and BglII sites. The vector is completed by inserting the HincII/RsaI 7.5 kD gene promoter fragment into the SmaI site of pUC8, excising the promoter as an EcoRI/BamHI fragment, and ligating it with the isolated EcoRI/SmaI/BamHI linker 25 fragment from pUC8 into EcoRI-digested vaccinia vector. Figure 13 also shows the nucleotide sequence in the region of the promoter/restriction site fragment junction for the CTP multimer embodiment.

In preparing the vectors of the invention, the 30 DNA encoding the desired immunogen is inserted into pGS20 using the restriction sites downstream of the promoter, as shown. The recombination vector is amplified in E. coli using transformation to Amp<sup>R</sup> and then coinfectd along with wild-type vaccinia into CV-1

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cells. Unlike other large DNA animal viruses, vaccinia transcribes and replicates its genome in the cytoplasm of infected cells. Many of the enzymes involved in its nucleic acid metabolism, such as DNA and RNA  
5 polymerases, enzymes to cap, methylate, and polyadenylate RNA, as well as thymidine kinase, are encoded in its own genome. Indeed, protein-free vaccinia is noninfective--since it encodes its own transcriptase and apparently cannot use the  
10 transcriptase used by its eukaryotic host, it cannot synthesize required proteins using its DNA alone in combination with the host cell machinery.

Use of the TK-encoding sequences to effect recombination is particularly desirable, as not only is  
15 this a nonessential portion of the vaccinia genome, but a selectable marker is provided for cells containing the recombined vaccinia--i.e., when the foreign DNA is inserted, the TK gene is inactivated, and the tk-recombinant viruses can be selected by plaque assay on  
20 tk- cells in the presence of 5-bromodeoxyuridine (BUdR). As shown in Figure 13, tk- recombinant virus plaques can be selected yielding the desired recombinant vaccinia. Additionally, tk- vaccinia have been shown to be  $10^5$  to  $10^6$  times less virulent in animals as  
25 compared to wild-type vaccinia, indicating the potential for an increased safety factor in humans.

While pGS20 is a convenient illustrative vector, it is understood that alternative constructions involving other vaccinia promoters in other nonessential  
30 regions of the gene may be used (Moss, et al, Gene Amplification Analysis, Vol. III, Pappas, T.K., et al, eds. (1982) New York, Elsevier, pp. 201-213; Mackett, M., et al, J Virol (1984) 586-864).

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The recombinant vaccinia can also be administered as a killed vaccine. Subject cells in tissue culture are infected with vaccinia in vitro, and then heat-killed. The attenuated, killed vaccinia are then administered with an adjuvant as are conventional vaccines.

G. Alternate Modes of Vaccine Construction and Administration

10       The autoantigenic constructions of the invention need not necessarily be administered as vaccinia borne DNA encoding them. They may also be produced as proteins using standard means of recombinant protein production and administered as conventional  
15 vaccines. The choice of route is dependent, of course, on the subject species and on the nature of the antigen.

Standard means for recombinant expression of DNA sequences are set forth below. In addition, the pGS20 vectors containing the desired autoantigen  
20 sequences may be used as expression vectors in vitro by using the transient expression which occurs when susceptible cells transfected with this vector are infected with vaccinia virus. The protein produced may be obtained from the cell lysate or from the  
25 supernatants, depending on whether construction of the vectors results or does not result in secretion.

If the autoantigens are administered as proteins, they are formulated into conventional dosage forms as injectables, either as solutions, suspensions,  
30 emulsions, or solids for reconstitution. Suitable excipients include water, saline, dextrose solution, glycerol solution, Hank's solution, Ringer's solution, and the like. Formulation techniques for protein containing vaccines are found in standard reference

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works such as Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, latest edition. Adjuvants may also be used, such as aluminum hydroxide, muramyl dipeptides, interferon, and immune-stimulating peptide regions. Vehicles can include squalene:aracel mixtures, croton oil, or other suitable compositions.

In those instances in which small peptides are used as autoantigens, such as, for instance, the peptides representing the non-homologous regions of LH, these are typically linked to carrier proteins, either at the protein or DNA level. Techniques for ligation at the DNA level are standard: mediation of the reaction using an appropriate ligase is used, as described below. At the protein level, a variety of chemistries can be used, including utilization of commercially available linkers such as SPDP and SMCC. The carrier protein in this case is an antigenically neutral peptide appropriate to the species, such as, for example, BSA for use in cattle, or HSA or tetanus toxoid for use in humans.

#### H. Production of Antibodies

As the vaccines of the invention are successful in raising antibodies which recognize epitopes associated with important regulators of metabolism, they provide a useful means to obtain components of immunoassays for such antigenic species. Also included in the invention, therefore, are antibody preparations obtained as a result of immunization with the vaccines provided. The antibody preparations may be obtained directly from the antisera as polyclonal preparations, or the peripheral blood cells or spleen cells from an immunized mammal may be used as fusion partners and monoclonal immunoglobulin producing hybridomas

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btained. For use in immunoassay, only the portion of the immunoglobulin related to specificity, and the invention therefore includes the Fab regions of the antibodies produced.

- 5           The antibody or Fab portion can be labeled or attached to solid support, or otherwise modified as is understood in the art in preparation for use in the assays.

10   Standard Methods

1.   Vector Construction

- Construction of suitable vectors containing the desired coding and control sequences employs standard  
15 ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

- Site specific DNA cleavage is performed by  
20 treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g., New England Biolabs,  
25 Product Catalog. In general, about 1 µg of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 µl of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate.  
30 Incubation times of about one hour to two hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from

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aqu ous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general  
5 description of size separations is found in Methods in Enzymology (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of E. coli DNA polymerase I (Klenow) in the presence of the four  
10 deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 min at 20 to 25°C in 50 mM Tris pH 7.6, 50 mM NaCl, 6 mM MgCl<sub>2</sub>, 6 mM DTT and 1 mM dNTPs. The Klenow fragment fills in at 5' sticky ends but chews back protruding 3' single strands, even though  
15 the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the sticky ends. After treatment with Klenow, the mixture is extracted with phenol/chloroform and  
20 ethanol precipitated. Treatment under appropriate conditions with S1 nuclease or mung bean nuclease results in hydrolysis of any single-stranded portion.

Synthetic oligonucleotides are prepared by the method of Efimov, V. A., et al (Nucleic Acids Res (1982)  
25 6875-6894), and can be prepared using commercially available automated oligonucleotide synthesizers. Kinasing of single strands prior to annealing or for labeling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 1  
30 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 1-2 mM ATP, 1.7 pmoles  $\gamma$ 32P-ATP (2.9 mCi/mmole), 0.1 mM spermidine, 0.1 mM EDTA.

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Ligations are performed in 15-50  $\mu$ l volumes under the following standard conditions and temperatures: 20 mM Tris-HCl, pH 7.5, 10 mM  $MgCl_2$ , 10 mM DTT, 33  $\mu$ g/ml BSA, 10 mM-50 mM NaCl, and either 40  $\mu$ M ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100  $\mu$ g/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations (usually employing a 10-30 fold molar excess of linkers) are performed at 1  $\mu$ M total ends concentration.

In vector constructions employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIP) in order to remove the 5' phosphate and prevent religation of the vector. Digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of  $Na^+$  and  $Mg^{+2}$  using about 1 unit of BAP or CIP per  $\mu$ g of vector at 60° for about one hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

## 2. Site-Specific Mutagenesis

For portions of vectors derived from cDNA or genomic DNA which require sequence modifications, site specific primer directed mutagenesis is used. This is conducted using a primer synthetic oligonucleotide complementary to a single stranded phage DNA to be

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mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the  
5 resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells which harbor the phage.

10 Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. The resulting plaques are hybridized with kinased synthetic primer and then washed at a temperature which permits  
15 hybrids of an exact match to remain, but at which the mismatches with the original strand are washed off. Plaques which remain hybridized to the probe at the stringent wash temperature are then picked, cultured, and the DNA recovered. Details of site specific  
20 mutation procedures are described below in specific examples.

For probing, plaques are screened by replicating the plaques onto duplicate nitrocellulose filter papers (S & S type BA-85) and infected cells are  
25 allowed to grow at 37°C for 14-16 hr on L agar containing 15 µg/ml tetracycline. The colonies are lysed with 10% SDS and the DNA is fixed to the filter by sequential treatment for 5 min with 500 mM NaOH/1.5 M NaCl, then 0.5 M Tris HCl(pH 8.0)/1.5 M NaCl followed by  
30 2 x standard saline citrate (SSC). Filters are air dried and baked at 80°C for 2 hr.

For synthetic (15-30 mer) oligonucleotide probes, the duplicate filters are prehybridized at 42°C for 2-8 hr with 10 ml per filter of oligo-hybridization



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buffer (6 x SSC, 0.1% SDS, 1 mM EDTA, 5x Denhardt's, 0.05% sodium pyrophosphate and 50 µg/ml denatured and sheared salmon sperm DNA).

5 The samples are hybridized with kinased oligonucleotide probes of 15-30 nucleotides under conditions which depend on the composition of the oligonucleotide. Typical conditions employ a temperature of 30-42°C for 24-36 hr with 5 ml/filter of this same oligo-hybridization buffer containing probe.

10 The filters are washed two times for 15 min at 23°C, each time with 6 x SSC, 0.1% SDS and 50 mM sodium phosphate buffer at pH 7, then are washed once for 2 min at the stringent wash temperature with 6 x SSC and 0.1% SDS. Typically, the stringent wash temperature for

15 oligonucleotides of 16-24 bases with from 1 to 3 mismatches will be 40-70°C, and can most easily be determined by successive washes of the hybridized filter. For example, the hybridized filters can be washed first at 40°C, then at 50°C, then at 60°C, and

20 then at 70°C, with air drying of the filter and autoradiography at -70°C overnight between each wash.

### 3. Verification of Construction

In the constructions set forth below, correct

25 ligations for plasmid construction are confirmed by first transforming E. coli strain MC1061 (Casadaban, M., et al, J Mol Biol (1980) 138:179-207) or other suitable host with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline

30 or other antibiotic resistance or using other markers depending on the mode of plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of Clewell, D. B., et al, Proc Natl Acad Sci (USA) (1969) 62:1159,

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opti nally f llowing chloramph nic l amplification  
(Clewell, D. B., J Bacteriol (1972) 110:667). The  
isolated DNA is analyzed by restriction and/or sequenced  
by the dideoxy method of Sanger, F., et al, Proc Natl  
5 Acad Sci (USA) (1977) 74:5463 as further described by  
Messing, et al, Nucleic Acids Res (1981) 9:309, or by  
the method of Maxam, et al, Methods in Enzymology (1980)  
65:499.

10 4. Hosts Exemplified

Host strains used in cloning and expression  
herein are as follows:

For cloning and sequencing, and for expression  
of construction under control of most bacterial  
15 promoters, E. coli strain MC1061 was used.

For M13 phage recombinants, E. coli strains  
susceptible to phage infection, such as E. coli strain  
JM101 are employed.

For eucaryotic expression, the monkey cell  
20 line, CV-1 is used.

5. Recombinant Expression Systems

Both procaryotic and eucaryotic systems may be  
used to express the autoantigen encoding sequences;  
25 procaryotic hosts are, of course, the most convenient  
for cloning procedures. Procaryotes most frequently are  
represented by various strains of E. coli; however,  
other microbial strains may also be used. Plasmid  
vectors which contain replication sites, selectable  
30 markers and control sequences derived from a species  
compatible with the host are used; for example, E. coli  
is typically transformed using derivatives of pBR322, a  
plasmid derived from an E. coli species by Bolivar, et  
al, Gene (1977) 2:95. pBR322 contains genes for

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ampicillin and tetracycline resistance, and thus provides multiple selectable markers which can be either retained or destroyed in constructing the desired vector. Commonly used procaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the  $\beta$ -lactamase (penicillinase) and lactose (lac) promoter systems (Chang, et al, Nature (1977) 198:1056) and the tryptophan (trp) promoter system (Goeddel, et al Nucleic Acids Res (1980) 8:4057) and the lambda derived  $P_L$  promoter and N-gene ribosome binding site (Shimatake, et al, Nature (1981) 292:128).

In addition to bacteria, eucaryotic microbes, such as yeast, may also be used as hosts. Laboratory strains of Saccharomyces cerevisiae, Baker's yeast, are most used although a number of other strains or species are commonly available. Vectors employing, for example, the 2  $\mu$  origin of replication of Broach, J. R., Meth Enz (1983) 101:307, or other yeast compatible origins of replication (see, for example, Stinchcomb, et al, Nature (1979) 282:39, Tschumper, G., et al, Gene (1980) 10:157 and Clarke, L. et al, Meth Enz (1983) 101:300) may be used. Control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes (Hess, et al, J Adv Enzyme Reg (1968) 7:149; Holland, et al, Biochemistry (1978) 17:4900). Additional promoters known in the art include the promoter for 3-phosphoglycerate kinase (Hitzeman, et al, J Biol Chem (1980) 255:2073). Other promoters, which have the additional advantage of transcription controlled by growth conditions and/or genetic background are the promoter regions for alcohol dehydrogenase 2,

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isocytochrom C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, the alpha factor system and enzymes responsible for maltose and galactose utilization. It is also believed terminator sequences are desirable at the 3' end of the coding sequences. Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes.

It is also, of course, possible to express genes encoding polypeptides in eucaryotic host cell cultures derived from multicellular organisms. See, for example, Axel, et al, 4,399,216. These systems have the additional advantage of the ability to splice out introns and thus can be used directly to express genomic fragments. Useful host cell lines include VERO and HeLa cells, and Chinese hamster ovary (CHO) cells. Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, the commonly used early and late promoters from Simian Virus 40 (SV 40) (Fiers, et al, Nature (1978) 273:113), or other viral promoters such as those derived from polyoma, Adenovirus 2, bovine papilloma virus, or avian sarcoma viruses. The controllable promoter, hMTII (Karin, M., et al, Nature (1982) 299:797-802) may also be used. General aspects of mammalian cell host system transformations have been described by Axel (supra). It now appears, also that "enhancer" regions are important in optimizing expression; these are, generally, sequences found upstream or downstream of the promoter region in non-coding DNA regions. Origins of replication may be obtained, if needed, from viral sources. However, integration into the chromosome is a common mechanism for DNA replication in eucaryotes.

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Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, S.N., Proc Natl Acad Sci (USA) (1972) 69:2110, or the  $RbCl_2$  method described in Maniatis, et al, Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor Press, p. 254 and Hanahan, D., J Mol Biol (1983) 166:557-580 may be used for procaryotes or other cells which contain substantial cell wall barriers. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology (1978) 52:546, optionally as modified by Wigler, M., et al, Cell (1979) 16:777-785 may be used. Transformations into yeast may be carried out according to the method of Beggs, J.D., Nature (1978) 275:104-109 or of Hinnen, A., et al, Proc Natl Acad Sci (USA) (1978) 75:1929.

#### 6. Additional cDNAs

Additional constructs can be made taking advantage of homologies between the DNA sequences disclosed herein and the corresponding hormones in other species. Thus, RNA may be prepared from the corresponding gland, such as the pituitary for LH, in a previously uninvestigated species, for example, rabbit, and a cDNA library prepared and probed. Effective techniques for extraction for mRNA are known in the art and generally employ affinity chromatography with oligo dT to obtain polyadenylated messenger RNA. The RNA is then used to obtain a cDNA library, for example, by cloning into the phage vector  $\lambda$ gt10 (Hunyh, T.V., et al, in DNA Cloning Techniques, a Practical Approach (1984) D. Glover, ed) or into a phage vector library capable of expression in bacteria using  $\lambda$ gt11 as

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described by Young, R.A., et al, Proc Natl Acad Sci USA (1983) 80:1194. The resulting cDNA can then be probed with denatured DNA sequences derived from the hormone sequences disclosed herein, for example, bovine LH  $\beta$  chain or the dog LH sequence.

### Examples

The following examples are intended to illustrate the invention but not to limit its scope.

#### Example 1

##### Construction of Autoantigen-Encoding Sequences

###### A. CTP Multimers

The construction of the desired genes is shown in Figure 1. The major portion of the gene is isolated as the BstNI/Sau3A fragment encoding amino acids 117-141 (Fiddes, J.C., et al, Nature (1980) 286:684-687). The 5' end and 3' end of the gene are synthesized chemically in order to provide desired overhang for polymerization, and, if desired, codon alteration. For all of the CTP fragments illustrated, the middle portion and C-terminal portions are identical; differences, however, occur in the upstream segment, depending on whether the native sequence is used or that altered in the 110 position.

For each of the three constructs, the three pertinent fragments are ligated to obtain the  $\beta$ -hCG CTP monomer and the desired product is isolated using polyacrylamide gels. Multimers are obtained by self-polymerization and separation of the various multimers on gels, e.g., the 3-mer (MW = 12 kD); 4-mer (MW = 16 kD); 6-mer (MW = 24 kD), or larger multimers.

The desired CTP<sub>n</sub> constructs are blunt-ended using Klenow and the appropriate dNTPs and either

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amplified in E. coli using intermediate vectors (Example 3) before direct recombination into vaccinia or are cloned into secondary carrier protein-encoding sequences before amplification in E. coli and subsequent  
5 recombination into vaccinia.

#### B. GnRH Multimers

The DNA sequences of the desired genes are shown in Figure 2. The double stranded portion encoding  
10 the monomer is obtained by annealing the two complementary strands, which strands are synthesized employing standard automated techniques. The monomer is isolated on a gel, and confirmed to be of the correct size. Multimers are obtained by self-polymerization and  
15 separation of the various multimers on gels, e.g., the 8-mer (243 base pairs); 10-mer (303 base pairs); 15-mer (453 base pairs), or larger multimers.

The desired GnRH<sub>n</sub> constructs are blunt-ended using Klenow and the appropriate dNTPs and either  
20 amplified in E. coli using intermediate vectors (Example 3) before direct recombination into vaccinia or cloned into secondary carrier protein-encoding sequences before amplification in E. coli and subsequent recombination into vaccinia.

25

#### C. LH Multimers

The complete cDNA sequences encoding the  $\beta$  chains of both human luteinizing hormone (hLH) and of the bovine counterpart (bLH) are known (see Figures  
30 3 and 4), and chemical synthesis could therefore be used to construct the desired oligonucleotides. However the constructions described below utilize portions of such length that although direct chemical synthesis is possible, it is more convenient to obtain the cDNA

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encoding them from standard libraries in  $\lambda$ gt10 prepared from human and bovine pituitary mRNA. The libraries are prepared by standard methods using EcoRI linkers, and are probed using the  $\beta$ -hCG cDNA insert removed with HindIII from the pBR322 cloning vector disclosed by Fiddes, J, et al, Nature (supra). (The LH and hCG  $\beta$  peptides are highly homologous.) The cDNA inserts are then transferred from the lambda phage vectors to the EcoRI site of pBR322 to obtain the cloning vectors pH LH and pBLH for the human and bovine forms of the protein, respectively.

Similarly, cDNA encoding the  $\beta$  peptides of dog LH was obtained from a  $\lambda$ gt10 cDNA library prepared using dog pituitary mRNA as template. The library was probed with the same HindIII HCG  $\beta$  peptide described above, and a single clone containing the coding sequence from amino acid 18 to the stop codon after amino acid 121 was retrieved. This clone, in combination with another similarly retrieved from the library, permitted deduction of the entire amino acid sequence for dog  $\beta$  LH, as well as the 18 residue signal sequence preceding the N-terminal serine residue of the mature protein. The complete cDNA sequence encoding the mature protein and its signal peptide, along with the deduced amino acid sequence is shown in Figure 3b.

The single clone containing the sequence encoding amino acids 18-121 was retrieved under hybridization conditions which include 30% formamide at 42° followed by washing 2 times at 50°C in 2XSSC. The EcoRI insert in the  $\lambda$ gt10 clone was then moved into the EcoRI site of pBR322 to obtain the cloning vector pDLH for the dog protein.

In a manner similar to that described above for retrieval of dog LH, the cDNA encoding the cat protein



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is prepared from cat pituitary mRNA and cloned into pBR322 to obtain pcLH.

The multimers are prepared directly in the vectors. For the bovine LH, a repeating sequence  
5 encoding amino acids 22-85 is inserted into the full length sequence in pbLH by repeatedly inserting the 192 bp fragment obtained by HaeIII digestion of pbLH into the StuI site which coincides with the HaeIII site at the codon for amino acid 22 (see Figure 3). While the  
10 blunt ended HaeIII fragment can be inserted in two possible orientations, the correct construction is distinguishable by restriction analysis, as it produces an EcoRI/StuI digestion fragment 192 bp shorter than the incorrect construction. The HaeIII fragment insertion  
15 is repeated for the desired number of (n-1) times, resulting in the 22-85 region peptide repeated n times (the StuI digested vector provides the additional repeat). Preferred values for n are 3-10.

For the human multimers, the EcoRI insert of  
20 pbLH is first transferred to the EcoRI site of M13mp8 for site specific mutagenesis to provide an MaeIII site at amino acids 1-2 to complement that already present in the cDNA bridging the codons for amino acids 111-112. (MaeIII is obtained by the purification procedure of  
25 Schmid, K., et al, Nucleic Acids Res (1985)). The primer is CCGTAGGTCACCTGGCGAAGCC as shown in Figure 4. The single stranded mutagenized phage is then hybridized to universal primer, extended with Klenow and the four dNTPs (30 minutes at room temperature), and  
30 cleaved with MaeIII to obtain the desired fragment extending between the new MaeIII site at codons 1-2 of the mature sequence and the naturally occurring MaeIII site at 111-112. This fragment is ligated by self polymerization in a manner analogous to that described

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for CTP or GnRH above, frame and orientation are maintained in the polymerization. For consistency, it is considered that these products of polymerization contain n-1 repeats. The multimers are then inserted  
5 into the MaeIII cleaved pHLH to obtain a cloning vector having n repeats (the extra repeat being supplied by the host vector). Preferred values of n are 3-10.

The dog multimer is constructed using the cDNA region between codon 43 (a RsaI site) and codon 115 (a  
10 SmaI site). The multimer is prepared by repeatedly inserting the blunt RsaI/SmaI fragment into SmaI cleaved pDLH and its resultants. Insertion of the RsaI/SmaI fragment into the SmaI site of pDLH, when in the correct orientation, destroys the RsaI site of the insert and  
15 recreates the SmaI site at the 3' end, thus permitting the repetitive insertion. The multimerized region can then be released by digestion with RsaI/SmaI as the only SmaI site remaining is at the 3' end of the multimerized region. The relevant portions of the junction regions  
20 are shown in Figure 3b. As above, preferred values of n are 3-10.

## Example 2

### Preparation of Cloning Vectors

25 Figure 5 shows the construction of the cloning vector pVA1 derived from pKK223-3 (Brosius, J., et al, Proc Natl Acad Sci (USA) (1984) 81:6929-6933). Construction of pVA2, derived from pKT19 and pKT41 (Talmadge, K., et al, Gene (1980) 12:235-241), is also  
30 shown in Figure 5.

Both constructions result in vectors which contain as an insert the synthetic nucleotide shown below:

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NcoI  
BamHI BalI NdeI SmaI  
 TGGATCCATGGCCAGGCATATGATTGATTGACCCGGG  
ACGTACCTAGGTACCGGTCCGTATACTAACTAACTGGGCCCTTAA  
 5      No PstI                              ter ter ter      EcoRI

This sequence, designated herein the NoPst/R oligomer, contains termination codons in all three possible reading frames, as well as convenient restriction NcoI, NdeI, and BalI sites for insertion of coding sequences. It also includes BamHI and SmaI sites at either end suitable for insertion into the pGS20 intermediate vector for vaccinia recombination.

In the constructions described, the NoPst/R oligomer is inserted into suitable host carrier vectors. The resulting cloning vectors ideally should lack NdeI and BalI sites to permit those contained in the NoPst/R oligomer to be used uniquely for subsequent insertion of the desired coding sequences; as BalI is a troublesome enzyme, the alternate upstream sites in the NoPst/R insert, the NcoI and BamHI sites, can be used in its place. The cloning vector also must carry markers, such as those conferring antibiotic resistance, as well as, preferably, a high copy number origin of replication. Two such vectors were constructed, pVA1, which fits the foregoing criteria but contains a HincII restriction site in the Amp<sup>R</sup> gene, and pVA2, which contains an intact Tet<sup>R</sup> gene and has no HincII or PvuII site, which sites are needed to be absent, as will be seen below, for certain manipulations of an inserted HA. Thus, initial cloning may be done in pVA1, but for constructions which require the use of HincII and PvuII sites, the HA gene must be moved into pVA2.

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### Construction of pVA1

Referring to Figure 5, pKK223-3 is first treated to remove unneeded DNA sequences and to remove an extraneous PvuII site. pKK223-3 is digested with EcoRI and PvuII, blunt-ended with Klenow, and then religated to generate pAL1, a 2.6 kb plasmid with the required origin of replication and the Amp<sup>R</sup> gene. pAL1 is then treated with EcoRI and PstI and ligated to the NoPst/R oligomer to obtain pVA1.

### Construction of pVA2

Referring to Figure 5, to prepare pVA2, pKT19 and pKT41 were digested with EcoRI and SphI, and the small pKT19 fragment isolated from a polyacrylamide gel was ligated to the large pKT41 fragment, also isolated from a 1% agarose gel, to obtain pKT1941. pKT1941 was digested with PvuII, ligated to a KpnI linker, cleaved with KpnI, and the religated to eliminate the PvuII site, creating pKT1941.1. The BalI and NdeI sites present in pKT1941.1 were eliminated in a two step procedure, the first step comprising digestion with NdeI, blunting with Klenow, ligating to KpnI linker, and religation of the plasmid; the second step analogously comprising digestion with BalI, ligating to KpnI linker, and religation to obtain pKT1941.3. This vector was digested with EcoRI and PstI and ligated with a tenfold molar excess of the synthetic fragment above to obtain pVA2.

### Example 3

#### Preparation of Intermediate Vectors for Intracellular Expression of Multimers

Intermediate vectors carrying the multimeric genes prepared above for intracellular expression when

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recombined into vaccinia are prepared by inserting the desired multimeric genes into the pVA1 or pVA2 vectors of Example 2. pVA1 or pVA2 is cleaved with NcoI and NdeI and blunt-ended with Klenow and the four dNTPs; the  
5 multimer-encoding sequences are correspondingly blunt-ended and ligated into the cleaved vector.

For the multimer of CTP, the synthetic polymerized multimers described in Example 1A are blunted and ligated into the NcoI(repair)/NdeI(repair)  
10 digested vectors. The resulting intermediate vectors, pVA1/CTP<sub>n</sub> and pVA2/CTP<sub>n</sub>, where n is the number of monomeric genes, are used as a source for the CTP<sub>n</sub> sequences in preparing recombinant vaccinia, as set forth below.

15 For the multimers of GnRH, self-polymerized, blunted GnRH DNA sequences of Example 1B are inserted analogously to obtain pVA1/GnRH<sub>n</sub> and pVA2/GnRH<sub>n</sub>.

For the multimers of the LH gene portions, suitable forms of the multimers can be inserted into the  
20 NcoI(blunt)/NdeI(blunt) vectors to provide, for example, pVA1/hLH<sub>n</sub> and pVA2/bLH<sub>n</sub>. However, in both cases, a properly placed NcoI site is needed, as shown in Figures 3 and 4, to place the upstream portion in reading frame with the start codon provided by the repaired NcoI from  
25 the vector. The multimer inserts are therefore removed from pbLH and phLH multimer-containing vectors by EcoRI digestion and placed into the EcoRI site of M13mp8 for site specific mutagenesis, the replicative forms obtained, and the NcoI(single strand  
30 removed)/EcoRI(blunt) fragments removed for insertion into pVA1 or pVA2.

The above vectors can be referred to generically as pVA/Hormone. The hormone monomers or multimers, preceded by an ATG start codon obtained by

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repair of the Nc I site and terminated by the termination codons provided in the NoPst/R fragment are transferred to pGS20 as a BamHI/SmaI fragment. Transient recombinant expression may be obtained using  
5 cells transfected with the pGS20 derivative and infected with vaccinia, or the vaccinia recombinants from these cells may be used directly as vaccines. In addition, the monomer or multimer hormone fragments may be ligated into vectors carrying the coding sequences for other  
10 proteins, as described below.

#### Example 4

##### Preparation of Derivative Vectors Containing HA

Three HA-containing vectors were prepared.  
15 pHA1 is used for insertion of the multimeric sequences into the mutagenized antigenic sites of the HA gene. As described below, it is provided with blunt end-resulting cleavage sites in the antigenic regions by site directed mutagenesis. In the constructions for GnRH and CTP,  
20 which are described in detail, these sites are located between codons, so that the reading frame for these multimers, which also terminates at codon boundaries, is maintained. pHA2 permits insertion using native restriction sites to ligate these multimers to the  
25 membrane anchor and signal sequences, the restriction sites also provide blunt-end termination at codon boundaries. pHA3 permits use of the EcoRI sites in the HA gene for ligation of the LH sequences, which have an extra bp beyond the codon boundary at either end.  
30 The construction of pHA1 and pHA2 is shown in Figure 6. The HA gene from pSVL-HA8 or pSVE-HA3 (Gething, M.J., Nature (1981) 293:620-625) was excised using HindIII/BamHI digestion. The resulting fragment

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contains the entire gene. A map of the HA gene is shown in Figure 7.

As shown in Figure 6, the HA gene fragment was ligated into HindIII/BglII digested pW6, a modified pBR322 which retains Amp<sup>R</sup>, but which contains a linker providing the desired restriction sites in the region of the deleted Tet<sup>R</sup> resistance gene. (To prepare pW6, pBR322 was digested with EcoRI and NruI and ligated with an EcoRI/blunt synthetic oligonucleotide, designated herein R/NoNru, and shown in the Figure, which contains BamHI, HindIII, BglII, and SmaI sites.) The pHA1 vector therefore contains the entire HA gene framed by BamHI and SmaI sites.

Because the remaining backbone fragment containing the Amp<sup>R</sup> gene from pBR322 contains unwanted restriction sites, it can be replaced by BamHI/NdeI digested pVA2. The HA gene from pHA1 is excised as a BamHI/NdeI fragment, which contains all of the gene except the codon 611 and the stop codon; the stop codon in pHA2 is provided by the downstream portion of the NoPst/R oligomer of pVA2.

The pHA3 vector is prepared from pHA2 by digesting with EcoRI, blunting, and religating to eliminate the EcoRI site of the linker before insertion of the HA gene as above.

The pHA1 cloning vector containing the HA gene can be conveniently modified to receive the in-frame multimer fragments by creating the restriction sites shown below, except for the HpaI site, in the A and B antigenic regions. pHA2 can be modified to provide the HpaI site. This is accomplished through site-specific mutagenesis using the site-specific primers:

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5'-GTGTCTGGTAGGCCTTCATTTTTC-3', which provides a StuI site between codons 139 and 140 in the A antigenic regions, as shown (HA/Stu);

5'-GCGCGGTGTCGCGAAATCCATCAT-3', which provides an NruI site between codons 137 and 138 of the A antigenic region (HA/Nru);

5'-TCTGGCTGGTTAACGAAGGATC-3', which provides a HpaI site between codons 150 and 151 in the B antigenic region; and

5'-TCCCATTGATATCACAGAACAAA-3', which creates an EcoRV site between codons 182 and 183 of the B antigenic region (HA/RV).

The mutagenesis is conducted by excising the BamHI/SmaI insert from pHA1 or pHA2, ligating the insert into BamHI/SmaI-digested M13mp8 for the mutagenesis, then religating the altered phage inserts into the original vectors.

The exemplified restriction sites above are designed to provide for in-frame insertion of the CTP and GnRH multimers prepared as described herein. Alternate, analogous sites are required for insertion of the LH sequences described which are excised as fragments in different reading frames.

#### Example 5

##### Preparation of HA/Multimer Constructs

pHA/Nru, pHA/Stu, pHA/Hpa, and pHA/RV are digested with NruI, StuI, HpaI, and EcoRV respectively (all provide blunt-ended cleavage fragments) and ligated with the both the monomer and multimer blunted gene fragments of CTP or GnRH prepared in Example 1. As shown in Figure 8, these inserts are in correct reading frame with the HA coding sequence, since the enzymes digest between codons, and the vectors thus contain



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chimeric genes with the desired hormone derived epitopes encoded into the antigenic regions of HA. The vectors are designated, for example, pCTP<sub>n</sub>/HA/Stu, pGnRH<sub>n</sub>/HA/Nru, or phCTP<sub>n</sub>/HA/Hpa or generically, pHormone/HA/site. (It will be recalled that these particular restriction sites are not suitable for the illustrated LH multimers.) As the HA proteins are carried to the surface of the infected cell, expression of the chimeras recombined into vaccinia results in exposure of these epitopes at cell surfaces.

#### Example 6

##### Preparation of Intermediate Vectors Carrying Chimeras Between HA Signal Sequence and Membrane Anchor

An alternative approach to providing chimeras for cell surface proteins uses the HA signal and membrane anchor. The resulting expressed proteins are, like those of Example 5, carried to the surface of the infected cells and presented for immunogenic response. Referring to Figures 7 and 9, the HA gene insert has a blunt-end generating HincII (Sall) restriction site between codons 16 and 17 and a blunt-end generating PvuII restriction site between codons 450 and 451. Cleavage of the HA gene with HincII (Sall) and PvuII excises the major portion of the HA protein and permits insertion of the hormone derived monomer and multimers between the signal sequence and the membrane anchor. The HA gene as inserted into pHA2 or pHA3 is used, as this vector does not contain interfering restriction sites.

The preparation of the analogous vectors containing the LH sequences is as follows. For bovine LH, pHA3 is digested with EcoRI which cuts at codons 226-227 and 394-395 of the HA gene and upon blunting

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leaves a two base pair extension beyond the codon boundary at either end. The bovine LH sequence containing, if desired, the multimeric forms is digested with DdeI which cuts between codons 18 and 19 and  
 5 between 86 and 87 as shown in Figure 3 and which leaves an additional base pair extending beyond the codon boundary at either end. Therefore, ligation of the bLH sequences into the vector prepared as above places the bLH coding sequences into reading frame with those of  
 10 the HA gene.

In a similar manner, pHA2 is digested with HincII (see Figure 7) which cleaves between codons 16 and 17 at the codon boundary and in codon 208 to leave one base pair extending beyond the codon boundary of  
 15 codon 209. This permits ligation of the human monomeric or multimeric  $\beta$ LH DNA which is excised from the cloning vector by digestion with NcoI and StuI to obtain the portion spanning codon 1 through all but the last base pair of codon 115. This places the human  $\beta$ LH cDNA in  
 20 reading frame with the HA sequences.

For dog  $\beta$  LH, the multimerized fragment from the pBR322 based vector of Example I is removed as a RsaI/SmaI blunted fragment and ligated into pHA2, which has been digested with SalI, blunted and then digested  
 25 with HincII. The relevant junction regions are:

```

      16      43      115      43      115      211
.....GTC GA || A CGA....GTC CC A CGA....CTC CC || A ACC.....
HA blunt SalI || Rsa   Rsa-Sma multimer   Sma || HA HincII site #2

```

30

Correct ligations are confirmed by restriction analysis. The desired immunogens are inserted in correct reading frame between the signal and membrane

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anchor in a series of vectors designated pCTP<sub>n</sub>/HAMB, pH<sub>n</sub>LH/HAMB, etc, or generically, pHormone/HAMB.

#### Example 7

5       Preparation of Intermediate Vectors Providing  
          Chimeras for Secretion of Hormone  
          Immunogen Using HA Signal

      Vectors providing for secretion may be constructed in three ways. First, the vectors  
10   constructed in Example 5, which contain hormone antigen in the antigenic regions of the HA carrier (Stu, Nru, Hpa, RV) can be further modified to delete the membrane anchor portion of the HA protein by using site-specific mutagenesis for deletion of the codons downstream of  
15   codon 514. Second, an NdeI/PvuII fragment between codons 451 and 510 may be deleted using restriction cleavage and religation for those vectors derived from pHA2. Third, a portion of HA lacking the membrane anchor can be used.

20       In the first approach, illustrated in Figure 10a, the entire coding sequence from pHormone/HA/Stu or the analogous Nru, Hpa, or RV vectors is excised as a BamHI/SmaI fragment and ligated into SmaI/BamHI-digested M13 for mutagenesis using the primer

25

5'-GGGGTAAAATTGAGCTGATTGATTGACCCG-3',

      which deletes the membrane anchor portion downstream of codon 504 as shown. The altered fragment is then  
30   religated into the large fragment obtained from BamHI/SmaI digestion of, for example, the starting, unaltered vector, pHA2. The resulting vectors are designated, generically, pHormone/HA<sub>504</sub>/site.

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In the second approach, shown in Figure 10b, vectors containing the monomer or multimer derived from pHA2 in Example 5, e.g. pCTP/HA/Stu or pGnRH/HA/RV, may be digested with PvuII and PstI to delete the desired region. The 3' overhang created by NdeI digestion is treated with Klenow in the presence of dCTP and the vectors are religated. Nucleotides 1601-1729 (Gething, et al, Nature (1980), supra) are deleted, fusing codon 450 to the last nucleotide of codon 511, and the translation proceeds in a new reading frame for another 8 codons and is then terminated by the third stop codon in the cloning vector. These vectors are designated, generically, pHormone/HA<sub>450</sub>/site.

Still another anchor-minus vector is constructed from an anchor-minus form of the HA gene from pSVEH20-A (Gething, M.J., et al, Nature (1982) 300:598-603) as the carrier protein, as shown in Figure 10c. The HA minus anchor portion is obtained as a BamHI/NdeI fragment for insertion into pVA2 by converting the downstream BamHI site in this partial gene to NdeI. This is accomplished by digesting pSVEHA20 with BamHI, treating with Klenow and the four dNTPs, and ligation to an NdeI linker. Subsequent digestion with BalI and NdeI provides the appropriate BalI/NdeI fragment for insertion into pVA1, to obtain pVA1/HA<sub>450-A</sub>. pVA1/HA<sub>450-A</sub> is subsequently modified by mutagenesis to accommodate the desired Hormone at the antigenic sites in a manner precisely analogous to that described for pVA1/HA in Example 5.

30

#### Example 8

##### Other Intermediate Vectors with Signal Sequences

The signal sequences from other proteins may also be used to construct intermediate vectors so that

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the ultimat recombinant vaccinia carrier will express hormone derived antigen as a secreted protein.

In one such construction, a synthetic gene encoding the bacterial alkaline phosphatase (BAP) signal sequence may be used. The construction of intermediate vectors carrying this sequence operably linked to multimer is shown in Figure 11.

The gene is synthesized using conventional techniques by construction of a series of complementary oligomers which hybridize to display compatible restriction sites, (ol5-ol20) as illustrated in Figure 11. The synthetic gene fragment having a partial NcoI 5' overhang and HindIII 3' overhang is ligated into a host vector, pSS1, which has been digested with NcoI and HindIII. pSS1 is a pBR322 derivative in which the synthetic oligonucleotides ol3 and ol4 shown in Figure 11 are ligated into the BamHI/EcoRI vector fragment of pBR322 to create a series of convenient restriction sites. The resulting vector, pSS2, is digested with HindIII and blunt-ended with Klenow, and then ligated to blunt-ended hormone derived fragments using a tenfold molar excess of the insert. The resulting vectors, pHormone/BAP, then contain the hormone antigen in reading frame with the BAP signal sequence and terminated with a reading frame in-frame stop codon and bounded by SmaI and BamHI sites provided by the oligomer fragment which had been inserted in pBR322. These intermediate vectors, pHormone/BAP, are then used as sources of the BamHI/SmaI insert for transfer into vaccinia.

The preprorenin signal sequence linked to the hormone antigen fragment may also be used. This construction is shown in Figure 12. As shown, the pSS1 is digested with HindIII, blunted with Klenow, and

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phenol extract d, digest d with NcoI, and the vector fragment isolated. The blunted hormone antigen is ligated to the vector fragment along with the renin signal sequence. The renin signal sequence is obtained as an NcoI/RsaI signal sequence-bearing fragment excised from pPP14. (pPP14 is extensively described in U.S. Serial No. 719,414, filed 3 April 1985, assigned to the same assignee and incorporated herein by reference; pPP14 carries the preprorenin cDNA insert). The ligation products are the pHormone/renin series, where the renin signal sequence is placed immediately upstream of the hormone antigen encoding gene, and the vector donates an in-frame stop codon (and two unimportant extra amino acids) immediately downstream of the hormone gene.

#### Example 9

##### Preparation of Vaccinia Recombinants

All of the intermediate vectors whose preparation has been described in Examples 3, 5-7, and 8 contain hormone antigen bearing cassettes which can be excised as BamHI/SmaI fragments for insertion into pGS20 by digesting the intermediate vectors with BamHI and SmaI, isolating the excised fragments on gels, and treating BamHI/SmaI-digested pGS20 with a tenfold molar excess of the isolated fragment. The resulting pGS20 derivatives contain the hormone antigen cassette in operable linkage to the vaccinia promoter and bounded by the portions of the TK gene, as shown in Figure 13.

These recombinant vectors are transfected into cells infected with wild-type vaccinia virus, which was purchased from Wyeth Laboratories, Inc. (Marietta, PA) and plaque purified twice in CV-1 cells. A small aliquot of virus stock (0.1 ml) is diluted with an equal

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volume of trypsin (0.25 mg/ml) and incubated for 30 min at 37°C with vortexing, followed by sonication to disperse any cell clumps. The virus is diluted to a concentration of  $5 \times 10^4$  plaque-forming units per ml in phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and penicillin/streptomycin. CV-1 cells are infected in monolayers on 60 mm plates with 1 ml vaccinia virus to give a multiplicity of infection of 0.05 pfu/cell. The virus inoculum is incubated on the cells for 2 hr at 37°C with rocking.

The DNA for transformation is prepared as described in Graham, et al, Virology (1973) 52:456; Stow, et al, J Gen Virol (1976) 33:447; and Frost, et al, Virology (1978) 91:39. Briefly, 5-10 µg of plasmid (pGS20 derivative) DNA, and 1-2 µg wild-type vaccinia virus DNA are added to 1 ml of Hepes-buffered saline (0.14 molar NaCl, 5 mM KCl, 1 mM Na phosphate, 0.1% dextrose, 20 mM Hepes, pH 7.05), and 50 µl of 2.5 molar  $\text{CaCl}_2$  is added. The solutions are mixed and left at room temperature for 30 min, and the desired precipitate of DNA forms in this time.

The virus inoculum is aspirated from the CV-1 cell layers, and 1 ml of the DNA precipitate is substituted and the layers left at room temperature for 30 min, after which 9 ml prewarmed Eagle's MEM containing 8% FBS is added. The layers are incubated for 3.5 hr at 37°C, before aspirating off the medium and replacing it with 10 ml fresh Eagle's MEM containing 8% FBS. The monolayers are then left at 37°C for 2 days, resulting in the development of vaccinia cytopathic effects. The cells and virus are harvested by scraping, spun down, and resuspended in 0.5 ml Eagle's MEM and frozen at -20°C.

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The resuspended virus/cell pellets are freeze-thawed three times by freezing the cells at -20°C for 30 minutes and quickly thawing them at 37°C, followed by sonication for one minute to disperse the virus/cell clumps. The resulting crude virus stock is then inoculated in serial 10-fold dilutions onto 143 cells (Mackett, M., et al, J Virol (1984) 49:857-864), a human tk- cell line. After two hours at 37°C, the 143 monolayers are then overlaid with 1% agarose containing 1 x modified Eagle's medium, 5% fetal bovine serum, and 25 µg/ml 5-bromodeoxyuridine (BUdR). After incubation for two days at 37°C, BUdR-resistant plaques are picked and grown for 48 hours at 37°C in 24 well plates of 143 cell monolayers in the presence of 25 µg/ml BUdR.

Virus is harvested and assayed for the presence of the hormone antigen gene by DNA-DNA dot blot hybridization, as follows: cells are scraped from the dish into an Eppendorf centrifuge tube, centrifuged for 1 minute, and the cell pellet resuspended in 0.2 ml PBS. After freeze-thawing 3 times and sonicating as described above, the sonicate is applied to a nitrocellulose filter and air dried. A wild-type virus control is also spotted onto the same filter. The filter is then placed on paper soaked in (1) 0.5 M NaOH, (2) 1 M Tris-HCl, pH 7.5, and (3) 2 x SSC, for 5 minutes each. The filter is then baked at 80°C under vacuum for 2 hours. The baked filter is prehybridized for 1 hour at 42°C in 5 ml 50% formamide, 4 x SSC, 5 x Denhardt's solution, and 0.1 mg/ml sheared and boiled salmon sperm DNA. The filter is then hybridized overnight at 42°C to  $2 \times 10^7$  cpm of  $^{32}$ P-labeled probe containing the desired hormone, for example, the hCG CTP gene probe, in 5 ml prehybridization buffer with 10 mg/ml dextran sulfate added. The probe is prepared by isolating 5



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µg of hCG CTP gene insert from one of the hCG CTP gene intermediate vectors and nick translating the DNA using a commercially available nick translation kit. The filter is then washed twice for 30 minutes in 0.5 x SSC, 5 0.1% SDS, air dried, and autoradiographed overnight at -70°C.

Virus containing desired hormone gene inserts are infected onto monolayers of 143 cells and left until a confluent cytopathic effect is obtained. The culture 10 medium is then aspirated off and the cells lysed in 1% SDS, 0.1 M β-mercaptoethanol, 50 mM Tris-HCl, pH 7.8. The lysate is made 0.5 mg/ml in proteinase K, incubated 4 hours at 37°C, phenol extracted, ethanol precipitated, and analyzed by restriction enzyme analysis to show that 15 the vaccinia genomes contain the desired genes.

#### Example 10

##### Bioassay

Rabbits are used as subjects to assess the 20 ability of the recombinant vaccinia to raise titers of neutralizing anti-hormone antibodies in serum. The following describes the procedure for CTP encoding vaccines; however, other hormones can be assayed using, for example, GnRH, LH, or FSH in place of CTP. The 25 procedure is analogous, but the antigen component in each assay is the appropriate hormone.

Pairs of rabbits are inoculated with wild-type or recombinant virus by intradermal injection of  $1-2 \times 10^8$  plaque-forming units distributed in 2-3 sites on 30 the back. Rabbits are bled from their ears at days 0, 14, 28, and sera tested for the presence of anti-hCG neutralizing antibodies in a radioligand receptor assay as follows.

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Commercially available hCG is iodinated in a 100 µl reaction by the chloramine T method of Greenwood, F.C., et al (J Biochem (1963) 89:114-123) in a 100 µl reaction mixture containing 5 µg hCG, 1 mCi Na<sup>125</sup>I, 0.1 µg chloramine T, and 0.1 M NaPO<sub>4</sub>, pH 7.5. The mixture is incubated at room temperature for 10 minutes and the reaction stopped with the addition of 0.1 µg sodium metabisulfite. Purification of <sup>125</sup>I-hCG is performed by reverse-phase HPLC on a C<sub>18</sub> column.

The interstitial cells of rat testis are homogenized in phosphate-buffered saline (PBS) as described in Catt, K.J., et al (J Clin Endocrinol Metab (1974) 34:123-127). The homogenate is centrifuged at 1500 g for 50 min and the pellet resuspended in 10 ml PBS per testis. A saturation binding curve is performed by incubating 100 µl of testis homogenate, 50 µl of tracer I<sup>125</sup>-hCG (20,000 cpm) in PBS-bovine gamma globulin (1 mg/ml), and 100 µl of hCG at various concentrations 0.1 to 100 ng/ml in preimmune serum, including a no-hCG control. After incubation at 24°C for 18 hours, the receptor-bound tracer is quantified by filtration through albumin-soaked 0.45 µ cellulose membranes and counted in a Beckman Gamma 5000 gamma counter. The concentration of hCG that results in 80% binding to the testis homogenate is then used with the labeled tracer in subsequent assays. Sera are tested for neutralizing activity by incubation with 100 µl testis homogenate, 50 µl of tracer I<sup>125</sup>-hCG (20,000 cpm) in PBS-bovine gamma globulin (1 mg/ml), 50 µl of hCG at the concentration determined in the saturation binding assay above, and 50 µl of sera diluted from 1:10 to 1:5000, including a no-serum control. The samples are then incubated, and bound tracer isolated

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and counted, exactly as described for the saturation binding assay above.

The titer of neutralizing activity is the dilution of serum that inhibits 50% of the binding in this assay. The titer of sera from animals that have low-level immunoneutralization responses will be 1:5 to 1:50, moderate responders will have sera with titers of 1:50 to 1:250, and strong responders will have sera with titers of greater than 1:1000. Recombinant vaccinia directing the expression of intracellular hCG antigens are more likely to produce low responses, and various secreted and membrane-bound antigens are more likely to produce moderate and strong responses. An alternate assay based on the change in weight of rat uterus can also be used.

The recombinant vaccinia which produce moderate to strong immunoneutralization responses in rabbits or show positive responses in the rat uterus weight assay will be tested for fertility control in baboons or rhesus monkeys. In general, the recombinant viruses that produce strong responses in rabbits will confer protection for longer periods of time, up to 3-5 years or more in humans, and the recombinant viruses that produce moderate responses in rabbits will protect for a shorter period of time, from 6 months to 3 years in humans.

#### Example 11

##### Preparation of Peptide Vaccines

The coding sequences for the autoantigens described above may also be expressed using conventional recombinant techniques, and the resulting peptides used as conventional vaccines. In one approach, the constructions in pGS20 which have been described in

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detail can be transiently expressed in CV-1 monkey cells using vaccinia virus coinfection, in the procedure of Cochran, M.A., et al, Proc Natl Acad Sci USA (1985) 82:19-23. Briefly, the CV-1 cells are infected with  
5 vaccinia at a moi of 30, and then transfected using calcium phosphate precipitation with the autoantigen-containing pGS20. The cells are harvested 12-48 hours after transfection, and the autoantigen recovered. If the autoantigen is membrane bound, the  
10 cells are solubilized; if secreted, the autoantigen is recovered from the medium.

In alternate approaches, the coding sequences for the hormones, either as multimers, fusion proteins with secondary carrier, or combinations thereof, are  
15 placed under control of conventional control sequences in expression vectors. For procaryotic expression, a particularly useful host vector is pKT52. pKT52 contains the "trc" promoter followed by an ATG and is constructed as follows below. The "trc" promoter  
20 contains the upstream portions of the trp promoter and the downstream, operator-containing, regions of the lac promoter.

To prepare pKT52, pKK233-2 (Amann, E., et al, Gene (1985) 40:183-190) was digested with EcoRI and  
25 PvuII, filled in with dATP and dTTP, and religated to obtain the correct construction pKT52.

pKT52 contains the desired trc promoter, a downstream ATG start codon, and downstream NcoI, PstI and HindIII sites. The autoantigen containing cassette  
30 can be excised from any of the vectors set forth above and inserted into NcoI/HindIII digested pKT52.

For eucaryotic expression, a useful host vector is pMT, which contains a convenient EcoRI site, and its

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relat d v ctor pMT-Apo. These vectors ar constructed as follows.

The host vector, pMT contains the metallothionein II (hMTII) control sequences, as described by Karin, M., et al, Nature (1982) 299:797-802. It is obtained by ligating the promoter into pUC8 as follows.

Plasmid 84H (Karin, M., et al (supra)) which carries the hMTII gene was digested to completion with BamHI, treated with exonuclease Bal-31 to remove terminal nucleotides, and then digested with HindIII to liberate an 840 bp fragment containing nucleotides -765 to +70 of the hMTII gene (nucleotide +1 is the first nucleotide transcribed). The 840 bp fragment was isolated and ligated with HindIII/HincII digested pUC8 (Vieira, J., et al, Gene (1982) 19:259-268) and the ligation mixture transformed into E. coli MC1061. The correct construction of pMT was confirmed by dideoxy nucleotide sequencing.

In addition, a derivative of the pMT, pMT-Apo, containing C-terminal regulatory signals was also prepared. pMT-Apo harbors a portion of the human liver protein ApoA<sub>1</sub> gene (Shoulders, C. C., et al, Nucleic Acids Res (1983) 11:2827-2837) which contains the 3'-terminal regulatory signals. A PstI/PstI 2.2 kb fragment of ApoA<sub>1</sub> gene (blunt ended) was cloned into the SmaI site of the pMT polylinker region, and the majority of the ApoA<sub>1</sub> gene removed by digestion with BamHI, blunt ending with Klenow, digestion with StuI, and religation. The resulting vector contains roughly 500 bp of the ApoA<sub>1</sub> gene from the 3' terminus as confirmed by dideoxy-sequence analysis. The autoantigen is inserted as an NcoI/EcoRI fragment.

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Example 12Antigenicity of Recombinantly Produced Peptides

Intermediate vectors containing multimers of 2 copies of ser110 CTP, 2 copies of des110 CTP, or 3 copies of GnRH between the HincII and PvuII sites of the HA gene of pHA2 were constructed using the appropriate multimer as described in Example 6, to give pCTP<sub>2</sub>(ser110)/HAMB, pCTP<sub>2</sub>(nol10)/HAMB, and pGnRH<sub>3</sub>/HAMB, respectively. The BamHI/SmaI fragments containing the coding sequences for amino acids 1-16 and 451-551 of the HA gene bracketing the specified multimers were transferred to pGS20 as described above, and the resulting pGS20/Carrier/Hormone vectors transformed into vaccinia infected CV-1 cells as in Example 11. The proteins were labeled with <sup>35</sup>S methionine, recovered from the cell surfaces, and a portion of them immunoprecipitated with polyclonal sera raised against hCG or GnRH as appropriate. The labeled proteins and their immunoprecipitates were analyzed on a 12.5% Laemmli gel, and the results are shown in Figure 14.

Lanes 1 and 14 of Figure 14 show low and high molecular weight standard, lanes 2-5 are total proteins from cells transformed with the vectors indicated on the figure. The high background levels did not permit proteins uniquely produced by the inserts to become apparent. However, immunoprecipitation with the appropriate antiserum permitted detection and verification of the molecular weights of these proteins.

Lanes 6 and 7 show the results of immunoprecipitation of membrane bound proteins of the CV-1 cells using anti-GnRH; lane 6 represents the pGS20/HA/GnRH<sub>3</sub> transformant, lane 7 is the pGS20 transformed control. The anti-GnRH is specific for the

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GnRH mid-region, as described by King, J.A., et al, Endocrinology (1980) 106:707-717. The smeared band at approximately 23 kd is of roughly the correct molecular weight (17 kd contributed by HA, 6 kd by the multimer) and may be a glycosylated protein (there are two glycosylation sites - both in the HA sequence).

Lanes 8-13 are immunoprecipitates with Miles antiserum (lanes 11-13) or Meloy antiserum (lanes 8-10), both raised against whole hCG in rabbits. Lanes 10 and 13 represent pGS20/HA/CTP<sub>2</sub>(ser110) transformants and show the presence of a new immunoprecipitated protein of approximately correct molecular weight (17 kd from the HA, 12 kd from the multimer). The control lanes 8 and 11, and the transformants with the noll0 multimer, lanes 9 and 12, do not show new bands.

Similar assays conducted on lysates of CV-1 cells infected with a recombinant vaccinia virus containing the DNA encoding the CTP 2-mer showed a band of correct molecular weight which specifically immunoprecipitated with anti-hCG antibody.

### Example 13

#### Construction of Species Specific Vaccines

Advantage is taken of the regions of low species homology in the LH sequences shown in Figure 15 to construct peptides based on these which are species-specific antifertility vaccines. Appropriate oligomers encoding the peptides represented by amino acids 58-69, 104-118 of the bovine sequence and 58-68 and 104-107 of the dog sequence, as shown in Figure 15 represent positions of low homology with human LH, thus permitting vaccines made from them to be handled safely by humans.

-74-

These oligomers may be polymerized and blunted  
to obtain multimers which are treated as described above  
to generate vaccines. As the peptides are relatively  
short, it may be advantageous to use them simultaneously  
5 to obtain a vaccine of maximum effectiveness.

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-75-

Claims

1. A vaccine effective in raising antibodies against a target indigenous protein which comprises a  
5 recombinantly produced immunogenic form of said protein, and wherein said immunogenic form is selected from the group consisting of:

a) a multimer which consists essentially of at least 2 repeating units of at least one epitope of said  
10 indigenous protein.

b) at least one epitope of said indigenous protein conjugated to a non-bacterial polypeptide, and

c) a multimer which consists essentially of at least 2 repeating units of at least one epitope of said  
15 indigenous protein conjugated to an additional polypeptide sequence.

2. A DNA sequence which encodes the immunogenic form protein of claim 1.  
20

3. A recombinant host cell which is transformed with the DNA of claim 2.

4. The DNA sequence of claim 2 which is  
25 disposed in a nonessential region of vaccinia virus.

5. The vaccine of claim 1 wherein the immunogenic form comprises a multimer which consists essentially of at least 2 repeating units of at least  
30 one epitope of said indigenous protein.

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6. The vaccine of claim 1 wherein the non-bacterial polypeptide is an influenza hemagglutinin.

5 7. The vaccine of claim 1 which is an antifertility vaccine effective in mammals.

8. The vaccine of claim 7 wherein the indigenous protein is selected from the group consisting  
10 of LH, GnRH, CG, and FSH.

9. A vaccine effective against mammalian fertility which comprises:

15 a vaccinia virus genome having disposed, in a nonessential region thereof, a DNA sequence having the formula Hormone<sub>n</sub>, wherein n is an integer equal to 1-20 and Hormone represents a DNA sequence derived from the sequence encoding a reproductive hormone.

20 10. The vaccine of claim 9 wherein the DNA sequence derived from the sequence encoding a reproductive hormone is selected from those derived from the group consisting of:

25 CTP where CTP represents a DNA encoding the carboxy terminal portion (CTP) of the  $\beta$ -peptide of human chorionic gonadotropin ( $\beta$ -hCG);

GnRH where GnRH represents a DNA encoding a peptide derived from gonadotropin releasing hormone;

30 hLH where hLH represents a DNA encoding a peptide derived from human luteinizing hormone;

bLH where bLH represents a DNA encoding a peptide derived from bovine luteinizing hormone;

dLH where dLH represents a DNA encoding a peptide derived from dog luteinizing hormone;

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cLH where cLH represents a DNA encoding a peptide derived from cat luteinizing hormone;

oLH where oLH represents a DNA encoding a peptide derived from ovine luteinizing hormone; and

5 FSH where FSH represents a DNA encoding a peptide derived from follicle stimulating hormone.

11. The vaccine of claim 10 wherein the DNA is selected from the group consisting of:

10 that which encodes the peptide consisting of amino acids 109-145 of  $\beta$ -hCG;

that which encodes the des<sub>110</sub> form of the peptide consisting of amino acids 109-145 of  $\beta$ -hCG;

15 that which encodes the ser<sub>110</sub> form of the peptide which is amino acids 109-145 of  $\beta$ -hCG;

that which encodes a peptide having the amino acid sequence of chicken II or human GnRH with a glutamine residue at the N-terminus;

20 that which encodes a peptide having the amino acid sequence of amino acids 2-111 of human LH;

that which encodes a peptide having the amino acid sequence of amino acids 22-85 of bovine LH; and

that which encodes a peptide having the amino acid sequence of amino acids 43-115 of dog LH.

25

12. The vaccine of claim 9 wherein the DNA of the formula Hormone<sub>n</sub> is operably linked to a signal sequence capable of effecting the secretion of a protein comprising Hormone<sub>n</sub>.

30

13. The vaccine of claim 9 wherein the DNA of the formula Hormone<sub>n</sub> is disposed in an antigen-encoding portion of a DNA encoding HA.

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14. A DNA sequence encoding a peptide effective in controlling mammalian fertility which comprises a DNA sequence encoding influenza hemagglutinin (HA) having disposed therein DNA of the formula Hormone<sub>n</sub>.

15. Antibodies or the Fab portions thereof raised against the vaccine of claim 1.

10

15

20

25

30

native

609	116
Thr	Gln
ACC	Phe
	Pro
Cys	Asp
TGT	GAT
ACA	CTA
	CTG
	GGG
	GCG
	AAG
	GT
	Bst

no110

1109	111								116
Thr	Asp	Asp	Pro	Arg	Phe	Gln			
ACC	GAT	GAC	CCC	CGC	TTC	C			
	CTA	CTG	GGG	GCG	AAG	GT			

ser110

	109	110	111		116
Thr	Ser	Asp	Asp	Pro	Arg
Ala	TCT	GAT	GAC	CCC	CGC
ACC	AGA	CTA	CTG	GGG	GCG
				AAG	GT
					BstNI

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Asp	Ser	Ser	Ser	Lys	Ala	Pro	Pro	Pro	Asp	Thr	Pro	
GAC	TCC	TCT	TCC	TCA	AGG	GCC	CTT	CCC	CCC	GAC	ACC	CC
CTG	AGG	AGA	AGG	AGT	TTC	CGG	GGA	GGG	GGG	CTG	TGG	GG [CTAG]
												Sau3A

142

Ile	Leu	Pro	Gln	
GATC	CTC	CCA	CAA	
	GAG	GGT	GTT	TGG

145

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no110 or  
ser110 A.

6

ACC-----BstNI | Sau3A TGG

ACC-----ACC-----TGG-----TGG-----

Fig. 1

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pig/cow/human: pyroGlu His Trp Ser Tyr Gly Leu Arg Pro Gly NH<sub>2</sub>  
 salmon: Trp Leu  
 chicken I: Gln  
 chicken II: His Trp Tyr

## GnRH SEQUENCE FROM VARIOUS SPECIES

### Human synthetic GnRH gene

Gln His Trp Ser Tyr Gly Leu Arg Pro Gly  
 CAG CAC TGG TCC TAT GGA CTG CGC CCT GGA  
 GTG ACC AGG ATA CCT GAC GCG GGA CCT GTC  
 Gln overhang

### Chicken II synthetic gene

Gln His Trp Ser His Gly Trp Tyr Pro Gly  
 CAG CAC TGG TCC CAC GGA TGG TAT CCT GGA  
 GTG ACC AGG GTG CCT ACC ATA GGA CCT GTC  
 Gln overhang

## GnRH GENES

# FIG. 2

**NUCLEOTIDE AND ENCODED AMINO ACID SEQUENCE OF DOG  $\beta$ LH CDNA**

**FIG. 3A** [superscripted numbers = (n-1)]

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**NUCLEOTIDE AND ENCODED AMINO ACID SEQUENCE OF DOG  $\beta$ LH cDNA**

												-18				-14
												Ala	Leu	Gln	Gly	Leu
												GCG	CTC	CAG	GGG	TTG
-13	Leu	Leu	Trp	Leu	Leu	Leu	Ser	Val	Gly	Gly	Val	Trp	Ala	Ser	Arg	
	CTG	CTG	TGG	CTG	CTG	CTG	AGT	GTG	GGT	GGG	GTG	TGG	GCA	TCC	AGG	
3	Gly	Pro	Leu	Arg	Pro	Leu	Cys	Arg	Pro	Ile	Asn	Ala	Thr	Leu	Ala	
	GGG	CCA	TTG	CGG	CCG	CTG	TGC	CGG	CCC	ATC	AAC	GCC	ACC	CTG	GCT	
18	Ala	Glu	Asn	Glu	Ala	Cys	Pro	Val	Cys	Ile	Thr	Phe	Thr	Thr	Thr	
	GCT	GAG	AAC	GAA	GCC	TGC	CCG	GTG	TGT	ATC	ACC	TTC	ACC	ACC	ACC	
33	Ile	Cys	Ala	Gly	Tyr	Cys	Pro	Ser	Met	Val	Arg	Val	Leu	Pro	Ala	
	ATC	TGT	GCC	GGC	TAC	TGC	CCC	AGC	ATG	<u>GTA</u>	<u>CGA</u>	GTG	CTG	CCA	GCC	
	RsaI															
48	Ala	Leu	Pro	Pro	Val	Pro	Gln	Pro	Val	Cys	Thr	Tyr	His	Glu	Leu	
	GCC	CTG	CCA	CCT	GTG	CCC	CAG	CCA	GTG	TGC	ACC	TAC	CAT	GAG	CTG	
63	His	Phe	Ala	Ser	Ile	Arg	Leu	Pro	Gly	Cys	Pro	Pro	Gly	Val	Asp	
	CAC	TTT	GCT	TCA	ATC	CGG	CTC	CCC	GGA	TGC	CCG	CCT	GGC	GTG	GAC	
76	Pro	Met	Val	Ser	Phe	Pro	Val	Ala	Leu	Ser	Cys	Arg	Cys	Gly	Pro	
	CCC	ATG	GTC	TCC	TTC	CCC	GTG	GCC	CTC	AGC	TGT	CGC	TGT	GGG	CCC	
93	Cys	Arg	Leu	Ser	Asn	Ser	Asp	Cys	Gly	Gly	Pro	Arg	Ala	Gln	Ser	
	TGC	CGT	CTC	AGC	AAC	TCC	GAC	TGT	GGG	GGT	CCC	AGA	GCT	CAA	TCC	
108	Leu	Ala	Cys	Asp	Arg	Pro	Leu	Leu	Pro	Gly	Leu	Leu	Phe	Leu		
	TTG	GCC	TGT	GAC	CGC	CCC	CTG	CTC	<u>CCG</u>	<u>GGC</u>	CTC	CTG	TTC	CTC	TAA	
	SmaI															

**GGATCCCCTCCTGCCCAACTCCTGGAGCCAGCAGATGCTCCTTCCCTCCCCTCCC**

AATAAAGGCTTCTAAACTGC<sub>polyA</sub>

**Join regions for insertion of RsaI-SmaI fragment into SmaI site:**

115	43	115	117
...Leu	Arg.....	Leu	Gly...
...CTC CC	A CGA.....	CTC CC	G GGC...
RsaI not recreated		SmaI recreated	

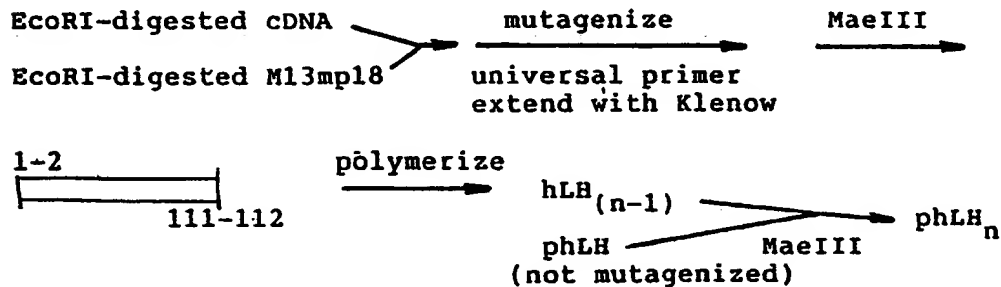
**FIG. 3B**

**SUBSTITUTE SHEET**



## NUCLEOTIDE AND ENCODED AMINO ACID SEQUENCE OF HUMAN $\beta$ LH

**Scheme to construct multimers of a portion of the  $\beta$  peptide:**



# SUBSTITUTE SHEET

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## Construction of Multimer Carrier Vectors

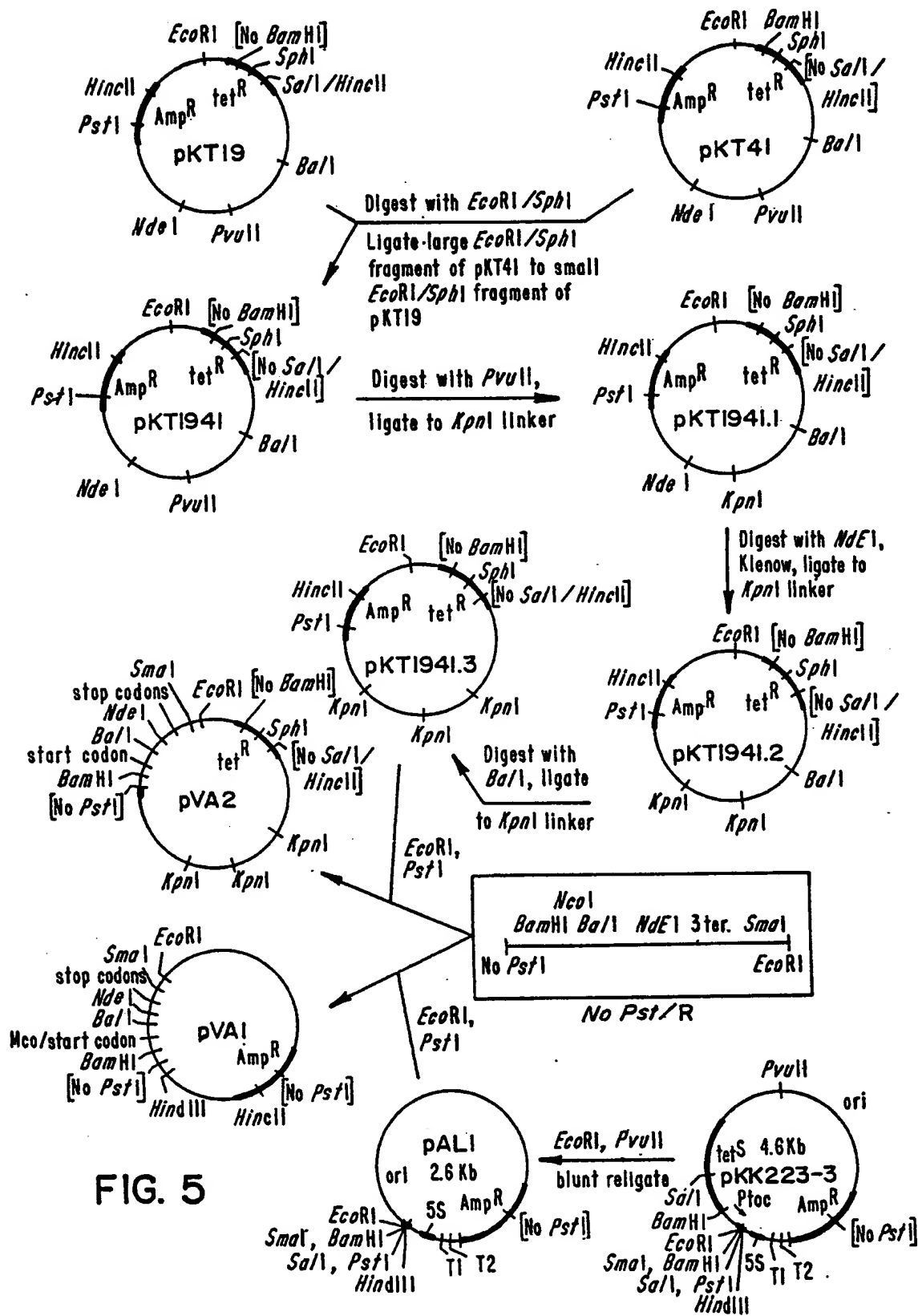


FIG. 5

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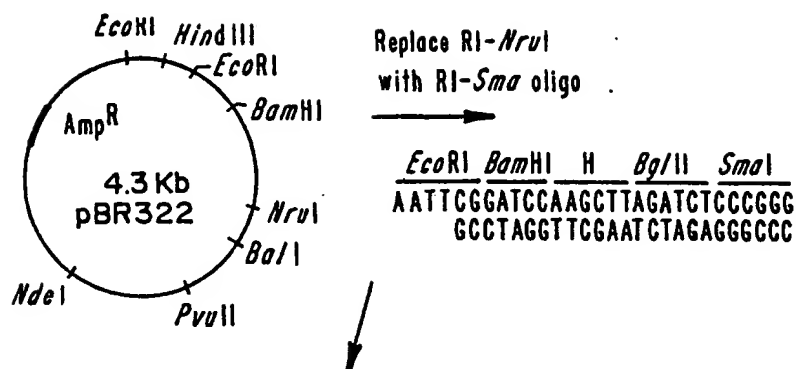
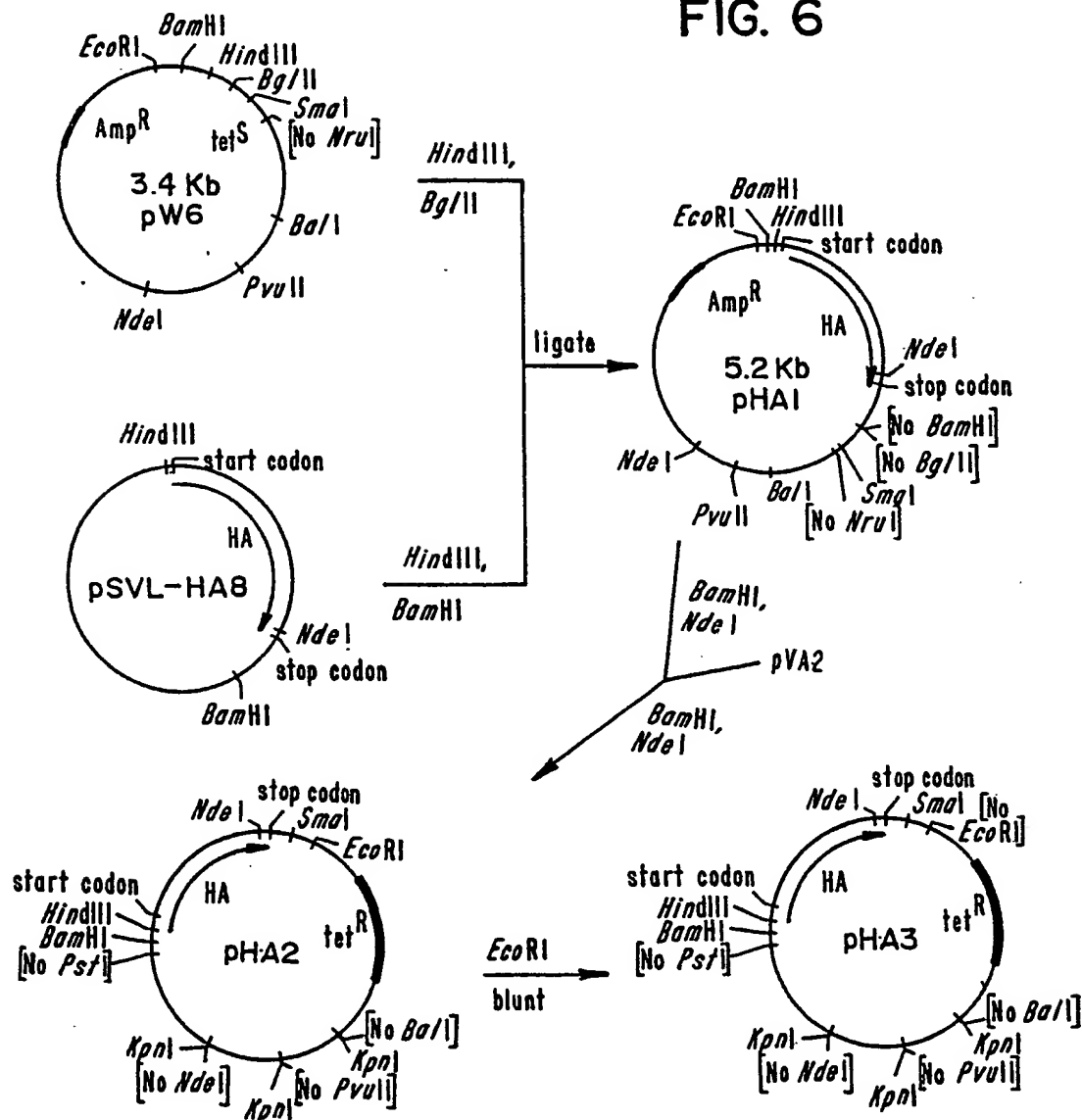
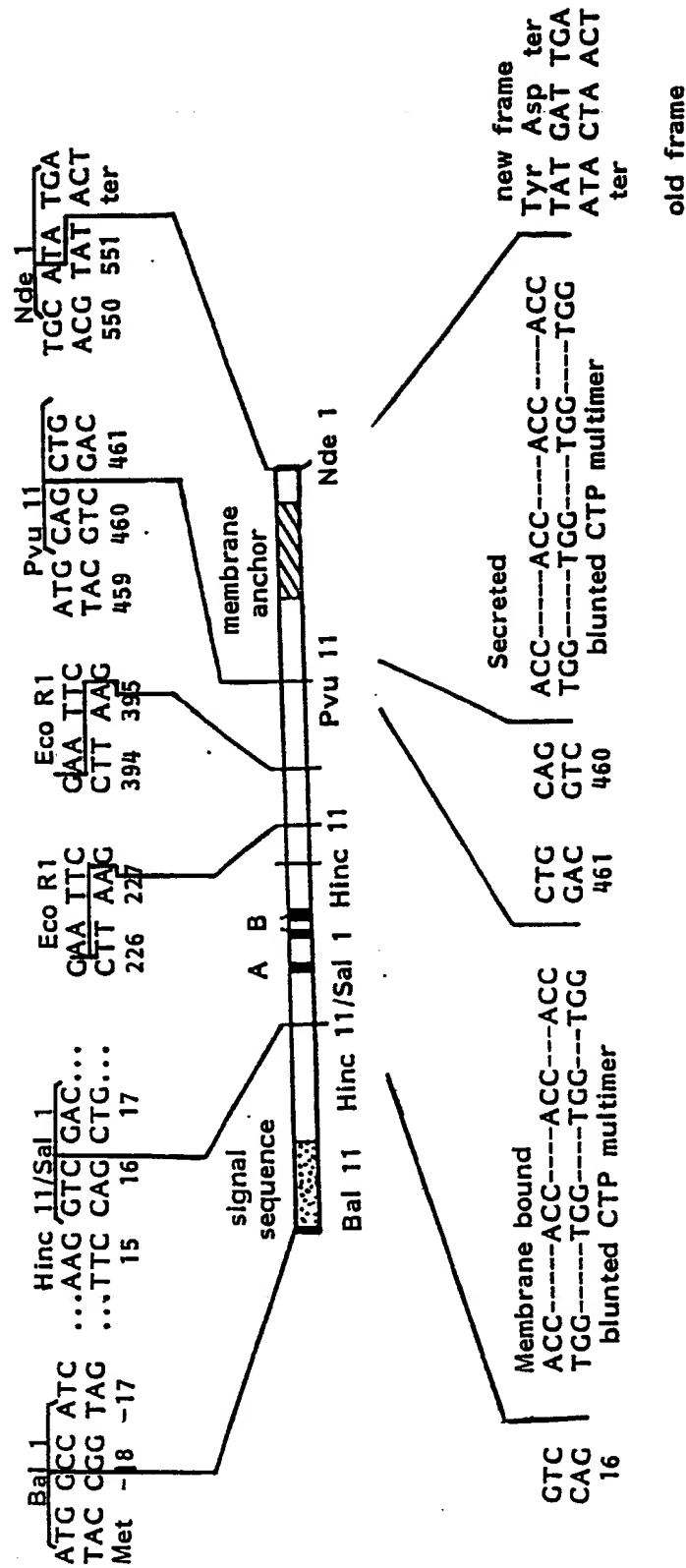


FIG. 6



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**FIG. 7** Schematic map of Hagene (middle), sequence of restriction sites important in cloning (above), and insertion site of membrane bound (bottom left) and secreting (bottom right) HA/hCG CTP fused polypeptides. The sequence of this antigenic variant, A/Japan/305/57 (H2) is from Gething, M.-J. et al., Nature 287,301,1980.

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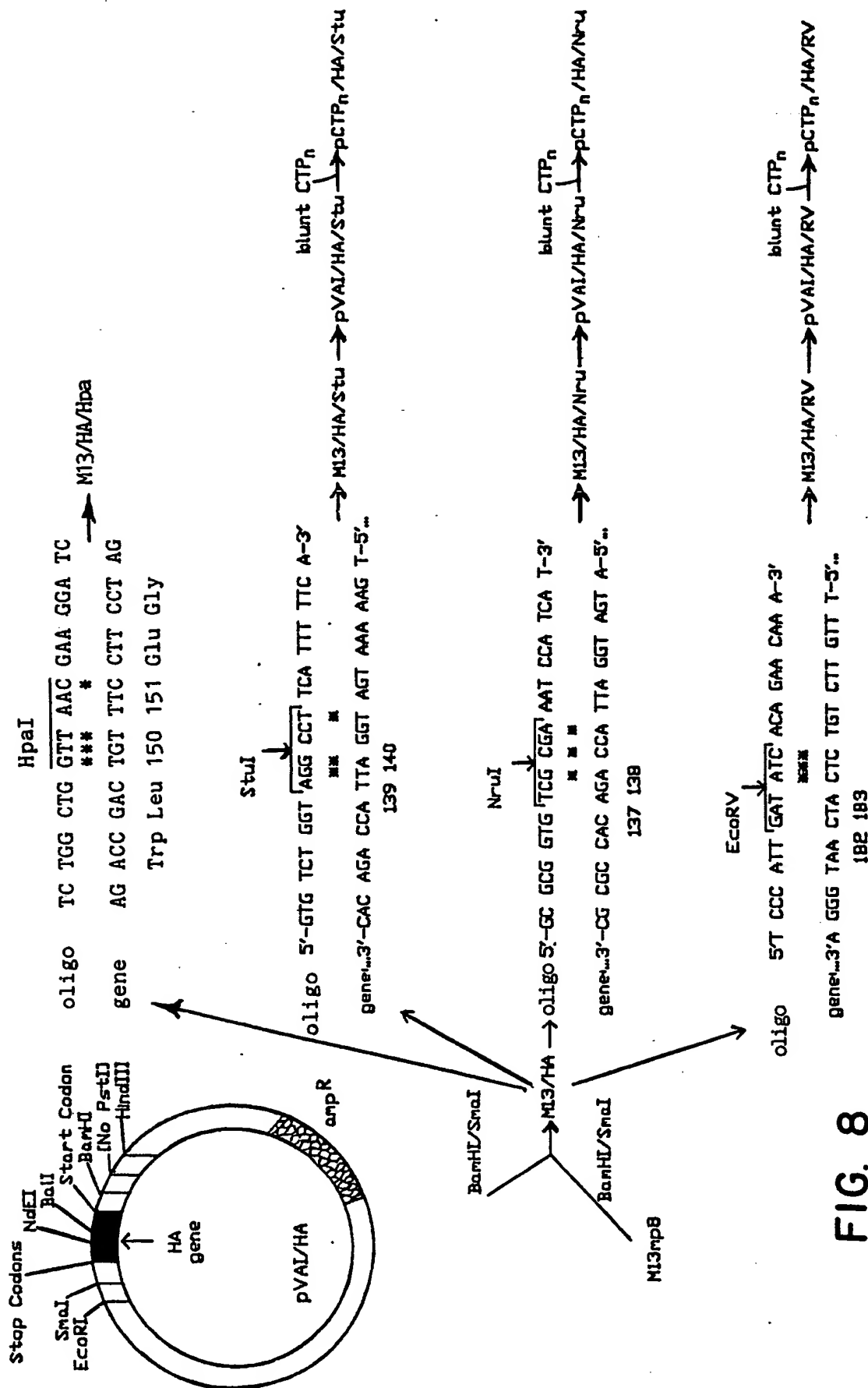


FIG. 8

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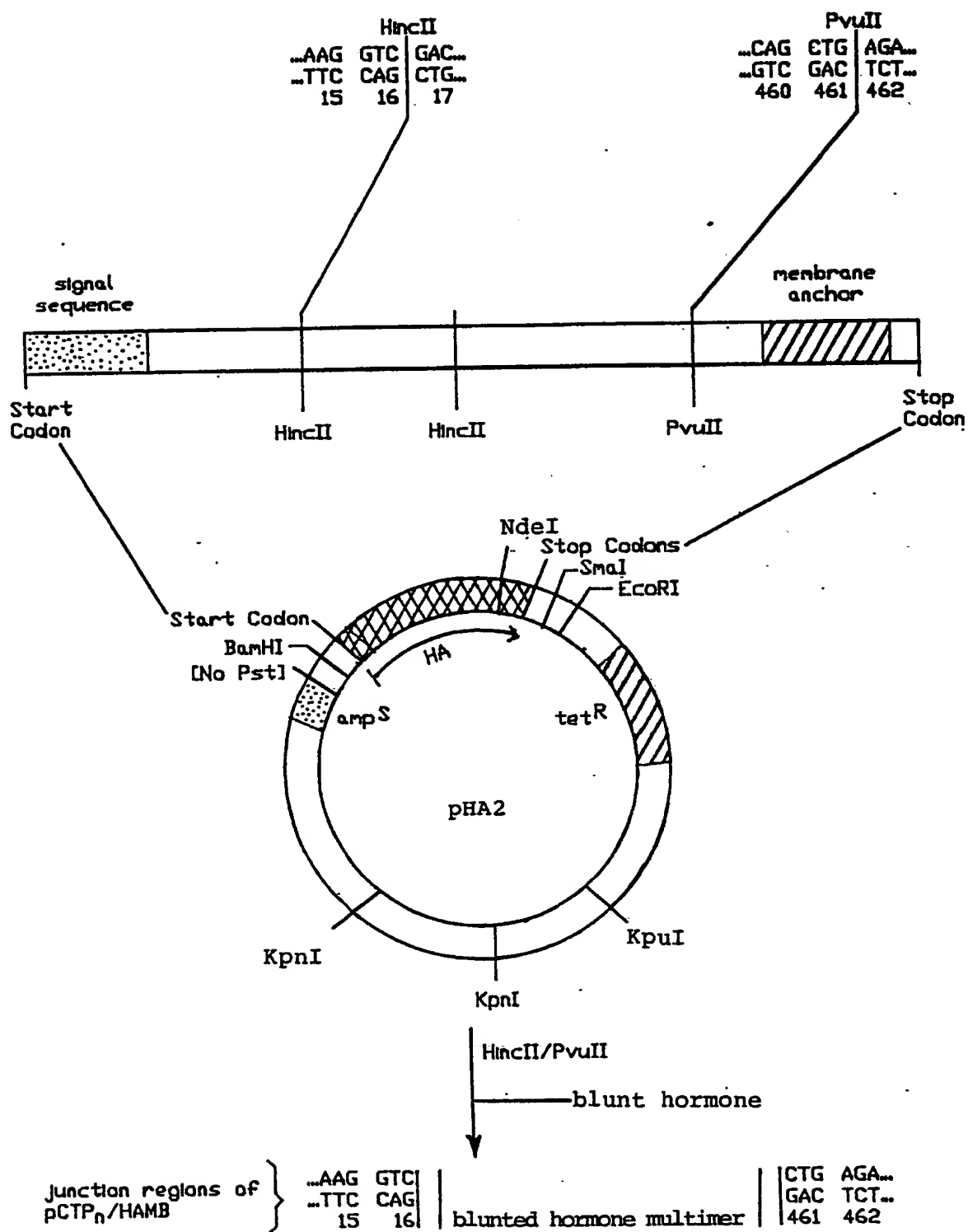


FIG. 9

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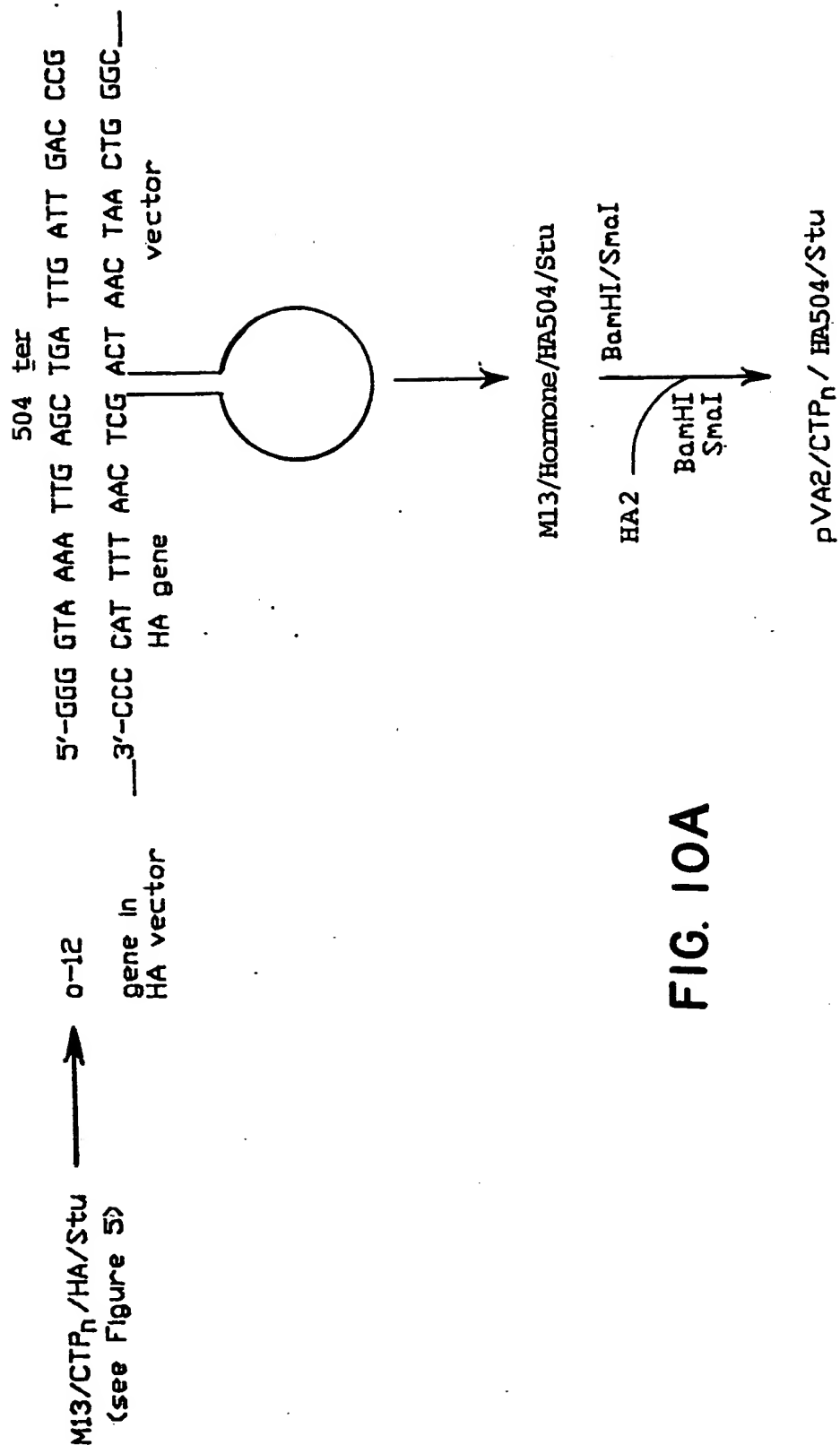


FIG. 10A





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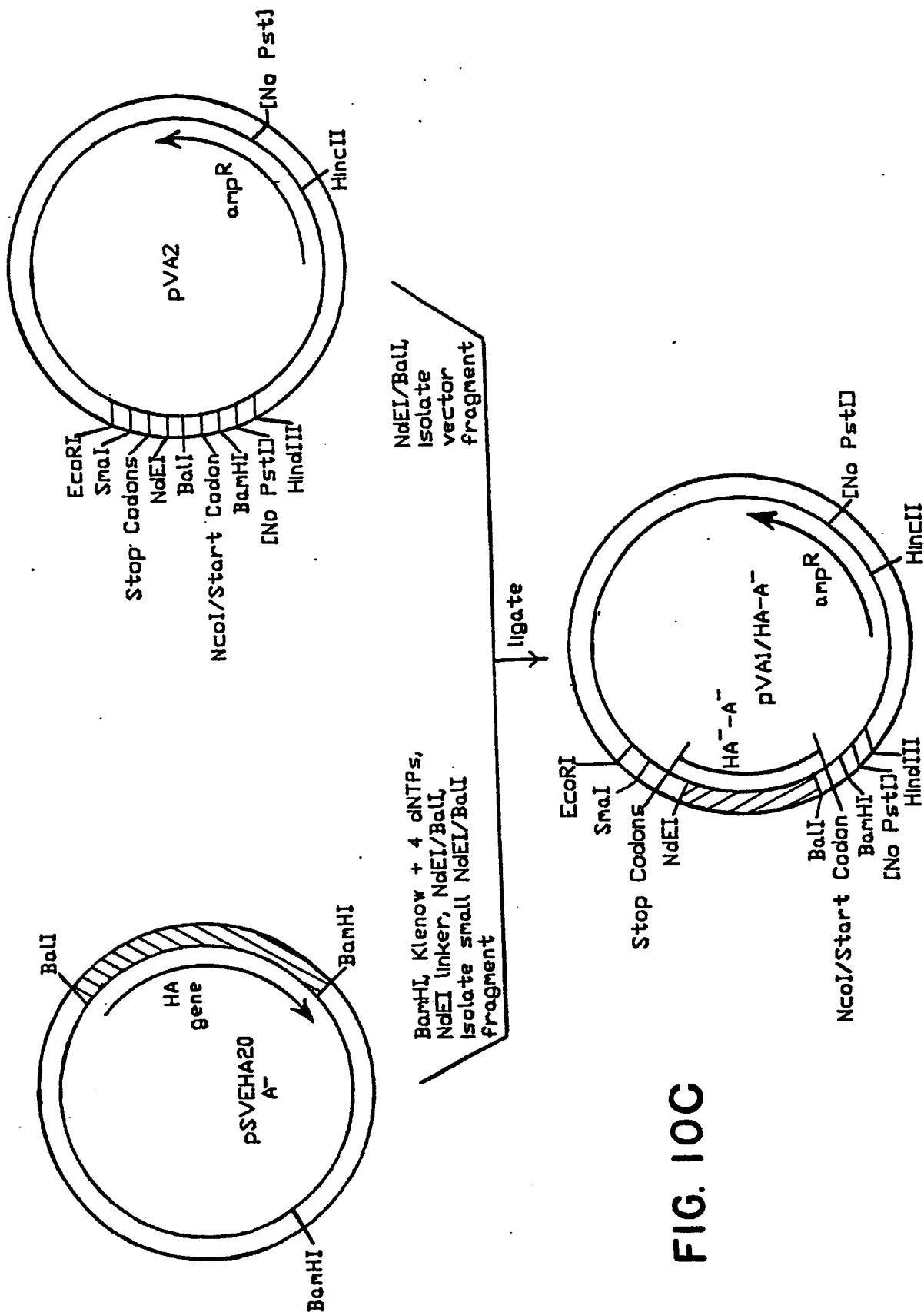
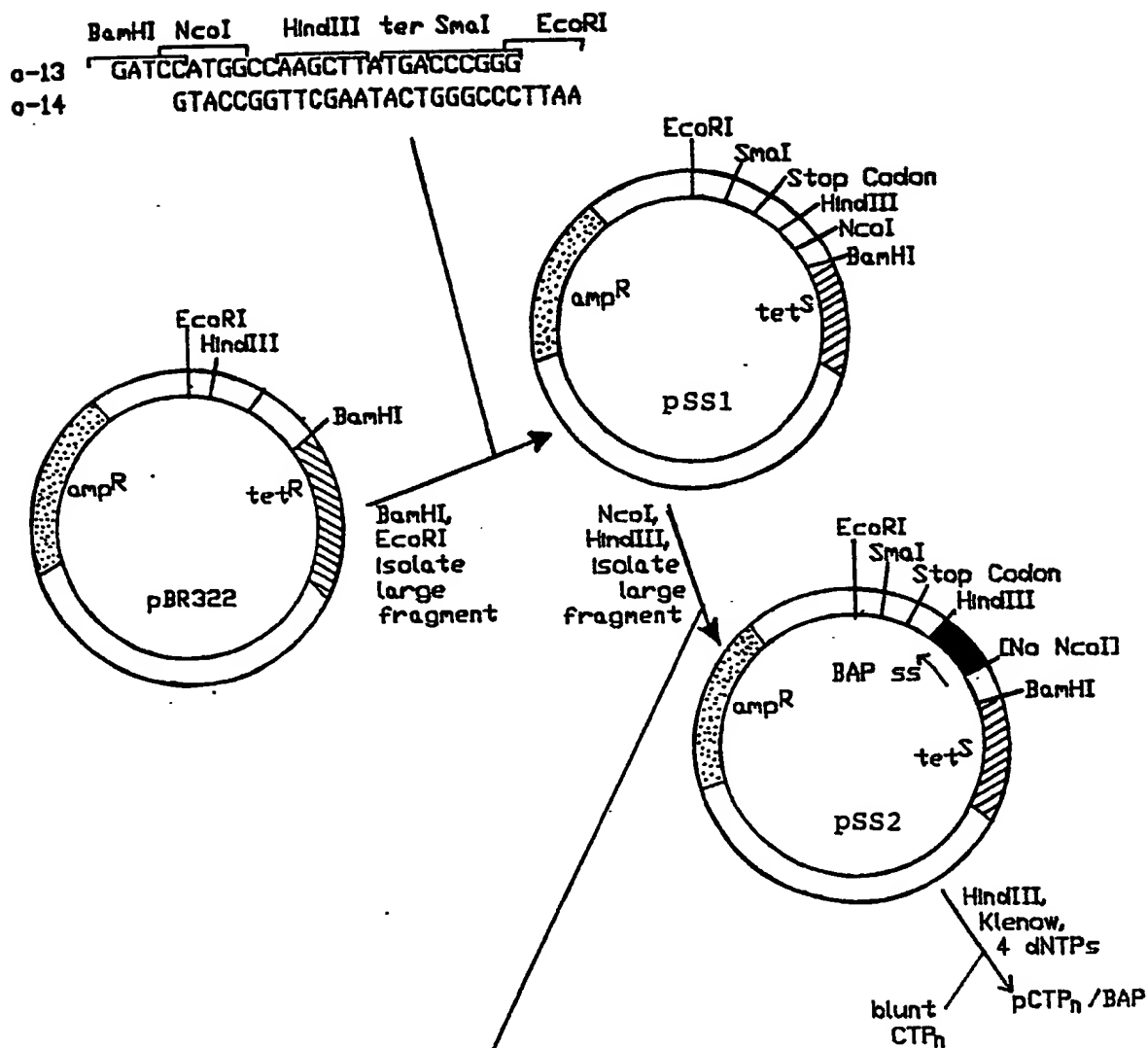


FIG. 10C

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	CAA GCT		Ser Leu ter
	GTT CGA		AGC TTA TGA
	blunted	blunted hormone	TCG AAT ACT
Junctions:	blunted		blunted
	HindIII		HindIII
	(vector)		(vector)

FIG. II

SUBSTITUTE SHEET

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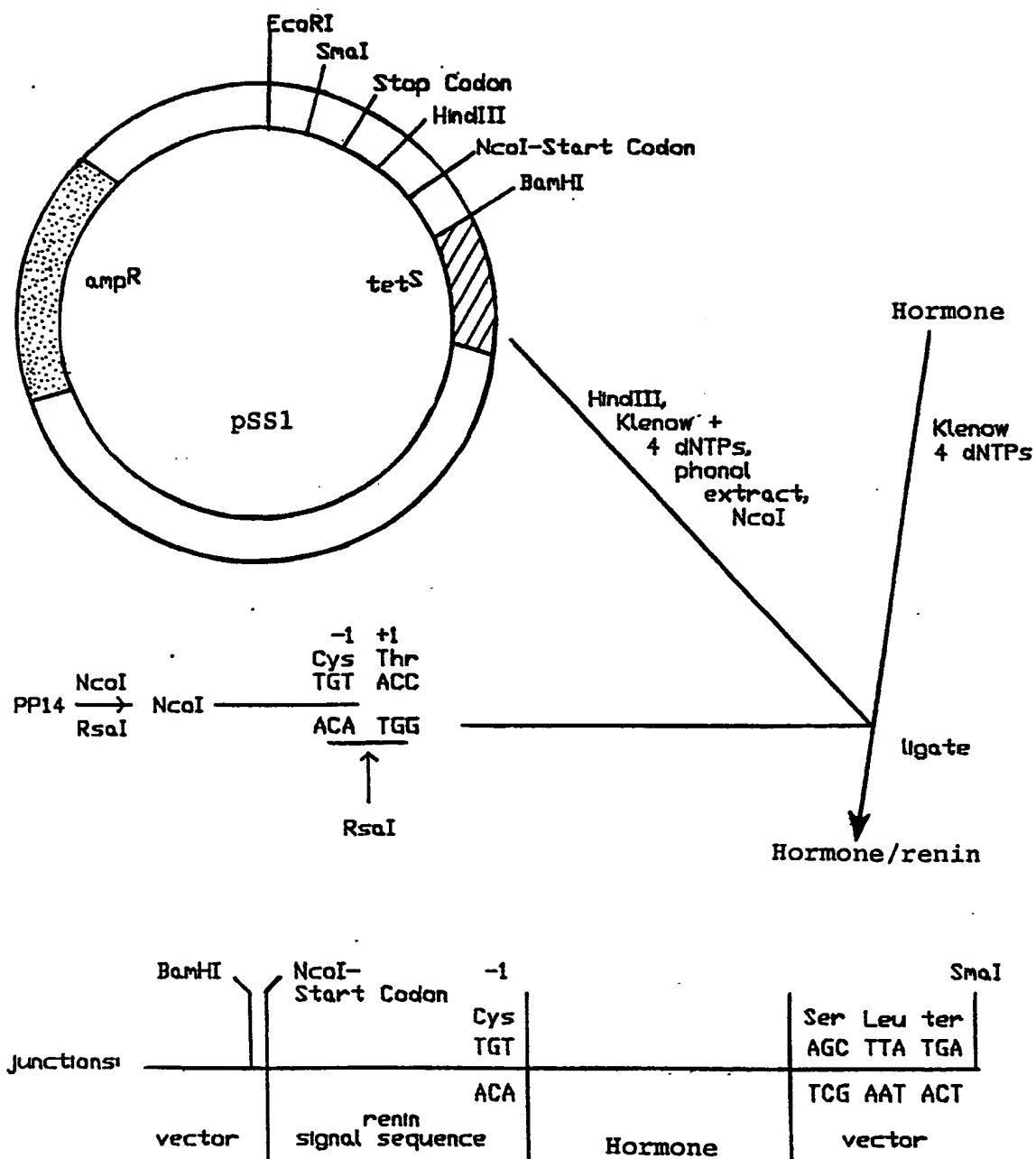
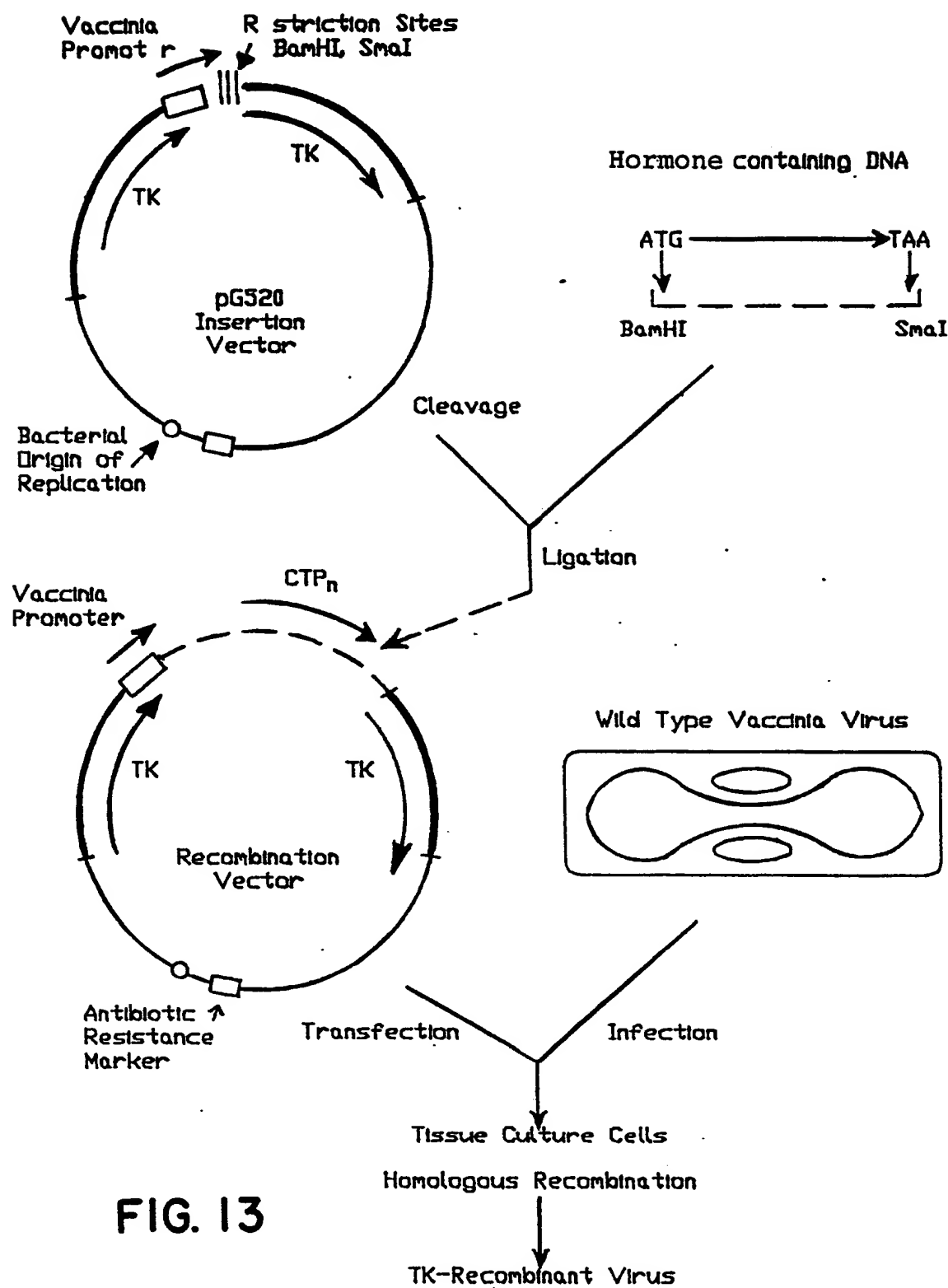


FIG. 12

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Scheme for construction of vaccinia virus  
recombinants expressing foreign genes.

**SUBSTITUTE SHEET**

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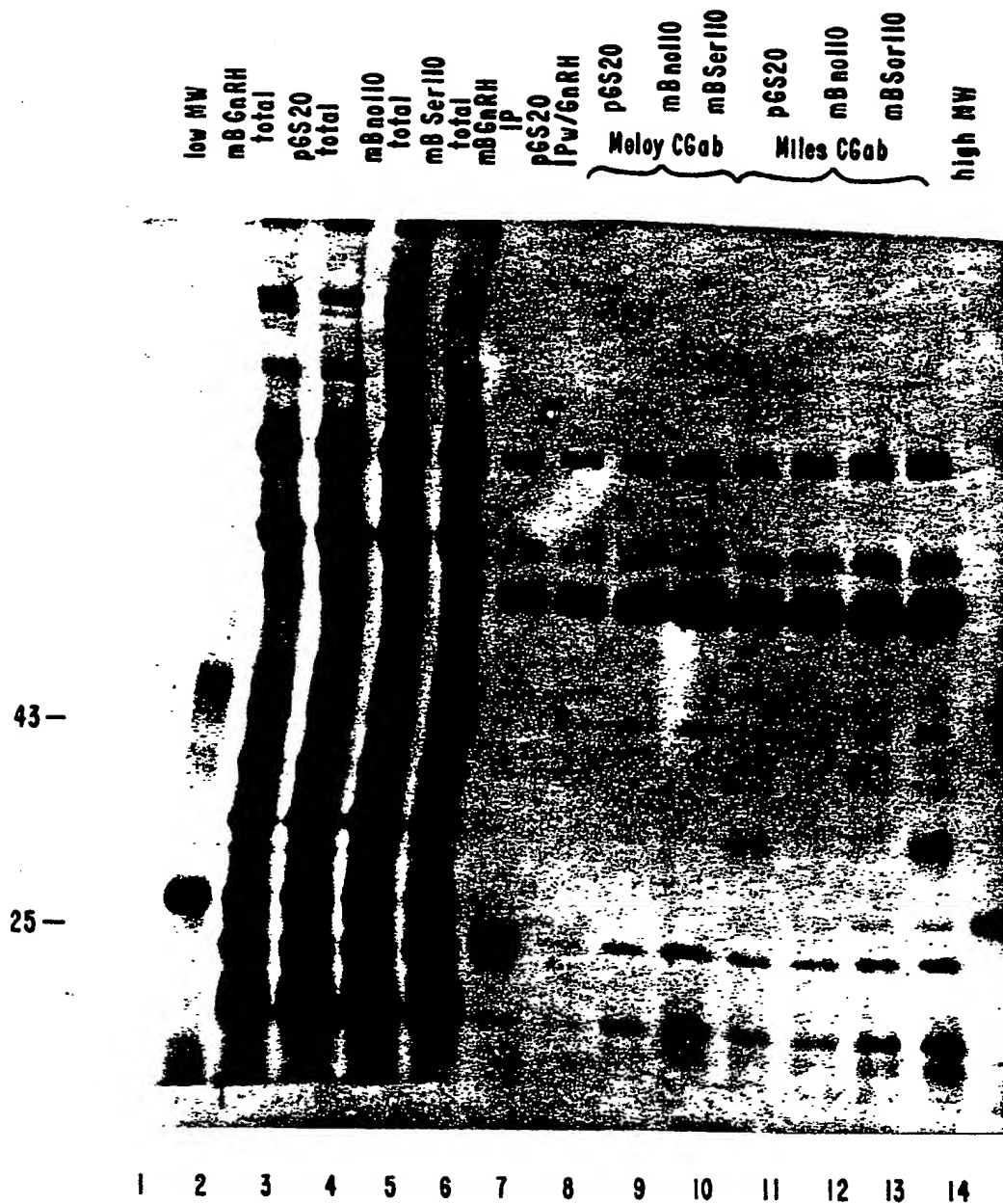


FIG. 14

### COMPARISON OF HUMAN, RAT, COW, AND DOG $\beta$ LH PROTEIN SEQUENCES

**FIG. 15A**

## **COW-SPECIFIC AND DOG-SPECIFIC $\beta$ LH PEPTIDES AND SYNTHETIC GENES**

**cowβLH( 58-69 ) :**

**[Ser110]-cowβLH(104-118):**

**DOG**

**dogβLH( 58-69 ) :**

**[Ser110]-dog $\beta$ LH(104-117):**

\* = difference with the human sequence

# SUBSTITUTE SHEET

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/01226

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC4: C12P 21/00, C12N 1/00; C07H 15/12, A61K 37/00, A61K 37/02						
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched <sup>4</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;">U.S.</td> <td style="padding: 5px;">           435/68, 172.3, 240, 253, 255, 317            536/27; 530/350+            424/85, 88; 935/9, 10, 32, 62         </td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>5</sup></div>			Classification System	Classification Symbols	U.S.	435/68, 172.3, 240, 253, 255, 317 536/27; 530/350+ 424/85, 88; 935/9, 10, 32, 62
Classification System	Classification Symbols					
U.S.	435/68, 172.3, 240, 253, 255, 317 536/27; 530/350+ 424/85, 88; 935/9, 10, 32, 62					
Computer Search CAS, Biosis, Lexis Vaccine for Contraception <u>DNA encoding Beta-HCG, Vaccinia Virus Vector</u>						
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>						
Category <sup>6</sup>	Citation of Document, <sup>15</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>				
Y	PCT, WO 85/01958 (REDDY) 9 May 1985. See entire document.	7-15				
Y	PCT, WO 85/01959 (REDDY) 9 May 1985. See entire document.	7-15				
Y	<u>Proc. Natl Acad. Sci. USA</u> (Washington, D.C., USA) Volume 82, issued June 1985 (Reddy et al) "Expression of Human Choriogonadotropin in Monkey Cells Using a Single SV40 Vector". See pages 3644-3648.	1-15				
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>*</sup> Special categories of cited documents: <sup>16</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>						
<b>IV. CERTIFICATION</b>						
Date of the Actual Completion of the International Search <sup>2</sup> <div style="text-align: center; font-size: 1.2em;">21 August 1986</div>		Date of Mailing of this International Search Report <sup>2</sup> <div style="text-align: center; font-size: 1.2em;">04 SEP 1986</div>				
International Searching Authority <sup>1</sup> <div style="text-align: center; font-size: 1.2em;">ISA/US</div>		Signature of Authorized Officer <sup>20</sup> <div style="text-align: center;">             Stephanie Seidman         </div>				



## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y

Fertility and Sterility  
(Philadelphia, Pennsylvania,  
USA) Volume 36, issued  
July 1981 (Stevens et al.)  
"Antifertility Effects of  
Immunization of Female Baboons  
with C-Terminal Peptides of  
the B-Subunit of Human Chorionic  
Gonadotropin". See pages  
98-106.

1-15

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>10</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>11</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No <sup>18</sup>
Y	<u>Proc. Natl. Acad. Sci. USA</u> (Washington, D.C., USA) Volume 80, issued December 1983 (Smith et al.) "Construction and Characterization of an Infectious Vaccinia Virus Recombinant that Expresses the Influenza Hemagglutinin Gene and Induces Resistance to Influenza Virus Infection in Hamsters". See pages 715-7159.	1-15
Y	<u>Nature</u> (London, England), Volume 302 issued April 1983 (Smith et al.) "Infectious Vaccinia Virus Recombinants that Express Hepatitis B Virus Surface Antigen". See pages 490-495.	1-15
Y	<u>Proc. Natl. Acad. Sci. USA</u> (Washington, D.C. USA) Volume 81 issued September 1984 (Tadeusz et al.) "Protection from Rabies by a vaccinia Virus Recombinant Containing the Rabies Virus Glycoprotein Gene". See pages 7192-7198.	1-15
A	<u>Journal of Andrology</u> (Birmingham, Alabama, USA) Volume 4 issued July 1983 (Schanbacher et al.) "Animal Model of Isolated Gonadotropin Deficiency". See pages 233-239.	1-15
Y	<u>Proc. Natl. Acad. Sci. USA</u> (Washington, D.C. USA) Volume 82 issued February 1985 (Talwar et al.) "Bioeffective Monoclonal Antibody against the Decapeptide Gonadotropin-Releasing Hormone: Reacting Determinant and Action on Ovulation and Estrus Suppression". See pages 1228-1231.	1-15
Y	<u>Proc. Natl. Acad. Sci. USA</u> (Washington D.C., USA) Volume 81 issued August 1984 (Shen et al.) "Multiple Joined Genes Prevent Product Degradation". See pages 4627-4631.	1-6

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**Summary**

<b>Document</b>	<b>Pages</b>	<b>Printed</b>	<b>Missed</b>	<b>Copies</b>
<b>WO008607383</b>	<b>102</b>	<b>102</b>	<b>0</b>	<b>1</b>
<b>Total (1)</b>	<b>102</b>	<b>102</b>	<b>0</b>	<b>-</b>



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 38/16</b>		<b>A1</b>	(11) International Publication Number: <b>WO 98/42365</b>
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(21) International Application Number: PCT/US98/06114 (22) International Filing Date: 27 March 1998 (27.03.98) (30) Priority Data: 60/041,009      27 March 1997 (27.03.97)      US 08/869,153      4 June 1997 (04.06.97)      US 60/057,456      3 September 1997 (03.09.97)      US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications US      60/041,009 (CIP) Filed on      27 March 1997 (27.03.97) US      08/869,153 (CIP) Filed on      4 June 1997 (04.06.97) US      60/057,456 (CIP) Filed on      3 September 1997 (03.09.97) (71) Applicant (for all designated States except US): BOARD OF SUPERVISORS OF LOUISIANA STATE UNIVERSITY AND AGRICULTURAL AND MECHANICAL COLLEGE [US/US]; c/o Louisiana Agricultural Experiment Station, LSU Agriculture Center Building, Room 104F, P.O. Box 25055, Baton Rouge, LA 70895-5505 (US).		(72) Inventors; and (75) Inventors/Applicants (for US only): ENRIGHT, Frederick, M. [US/US]; 5238 North Chalet Court, Baton Rouge, LA 70808 (US). JAYNES, Jesse, M. [US/US]; 2417 High Ridge Drive, Raleigh, NC 27606 (US). HANSEL, William [US/US]; 356 Kenilworth Parkway, Baton Rouge, LA 70808 (US). KOONCE, Kenneth, L. [US/US]; 6976 S. Fieldgate Court, Baton Rouge, LA 70808 (US). McCANN, Samuel, M. [US/US]; Apartment B, 7834 Jefferson Place Boulevard, Baton Rouge, LA 70809 (US). YU, Wen, H. [US/US]; Apartment 117, 4155 Essen Lane, Baton Rouge, LA 70809 (US). MELROSE, Patricia, A. [US/US]; 7446 Shrewsbury Avenue, Baton Rouge, LA 70808 (US). FOIL, Lane, D. [US/US]; 1180 Stanford Avenue, Baton Rouge, LA 70808 (US). ELZER, Philip, H. [US/US]; 10231 Azrok Avenue, Baton Rouge, LA 70809 (US). (74) Agent: RUNNELS, John, H.; Taylor, Porter, Brooks & Phillips, L.L.P., P.O. Box 2471, Baton Rouge, LA 70821-2471 (US). (81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report.	
(54) Title: LIGAND/LYTIC PEPTIDE COMPOSITIONS AND METHODS OF USE			
(57) Abstract			
<p>Amphipathic lytic peptides are ideally suited to use in a ligand/cytotoxin combination to specifically inhibit cells that are driven by or are dependent upon a specific ligand interaction; for example, to induce sterility or long-term contraception, or to attack tumor cells, or to selectively lyse virally-infected cells, or to attack lymphocytes responsible for autoimmune diseases. The peptides act directly on cell membranes, and need not be internalized. Administering a combination of gonadotropin-releasing hormone (GnRH) (or a GnRH agonist) and a membrane-active lytic peptide produces long-term contraception or sterilization in animals <i>in vivo</i>. Administering <i>in vivo</i> a combination of a ligand and a membrane-active lytic peptide kills cells with a receptor for the ligand. The compounds are relatively small, and are not antigenic. Lysis of gonadotropes has been observed to be very rapid (on the order of ten minutes). Lysis of tumor cells is rapid. The two components –the ligand and the lytic peptide– may optionally be administered as a fusion peptide, or they may be administered separately, with the ligand administered slightly before the lytic peptide, to activate cells with receptors for the ligand, and thereby make those cells susceptible to lysis by the lytic peptide. The compounds may be used in gene therapy to treat malignant or non-malignant tumors, and other diseases caused by clones or populations of "normal" host cells bearing specific receptors (such as lymphocytes), because genes encoding a lytic peptide or encoding a lytic peptide/peptide hormone fusion may readily be inserted into hematopoietic stem cells or myeloid precursor cells.</p>			

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**LIGAND/LYTIC PEPTIDE COMPOSITIONS AND METHODS OF USE**

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The benefit of the March 27, 1997 filing date of provisional application serial number 60/041,009 and of the September 3, 1997 filing date of provisional application 60/057,456 are claimed under 35 U.S.C. § 119(e) in the United States, and are claimed under applicable treaties and conventions outside the United States. The benefit of the June 4, 1997 filing date of United States non-provisional application 08/869,153 is claimed under 35 U.S.C. § 120 in the United States, and is claimed under applicable treaties and conventions outside the United States.

**TECHNICAL FIELD**

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This invention pertains to compositions and methods for specifically inhibiting cells that are driven by or are dependent on specific ligand interactions. Examples are compositions and methods for long-term contraception or sterilization; compositions and methods for inhibiting or killing malignant and non-malignant, hormone-dependent tumors; compositions and methods for selectively killing virally infected cells; and compositions and methods for selectively destroying lymphocytes responsible for autoimmune disorders.

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**BACKGROUND ART**

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Compositions that have sometimes been used for long-term contraception include those based upon natural or synthetic steroidal hormones to "trick" the female reproductive tract into a "false pregnancy." These steroidal hormones must be administered repeatedly to prevent completion of the estrous cycle and conception. Steroids have side effects that can be potentially dangerous.

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P. Olson *et al.*, "Endocrine Regulation of the Corpus Luteum of the Bitch as a Potential Target for Altering Fertility," *J. Reprod. Fert. Suppl.*, vol. 39, pp. 27-40 (1989) discusses the luteal phase and its regulation in bitches. The following discussion appears at page 37: "Specific toxins can be linked to an antibody or hormone and carried to a specific target cell (or cells) which is then killed by the toxin. The idea of developing a 'magic bullet'

has been discussed for decades but is now gaining renewed recognition as a potential, highly selective method for destroying specific tissues while leaving other tissues unharmed. For many years it was impossible to develop large quantities of antibodies which would react specifically with only single antigenic determinants. However, with the advent of monoclonal antibodies, this problem has been largely overcome. Antibodies can be developed to specific hormone receptors (such as the LH receptor) and then coupled to a toxin. All cells with LH receptors should then be destroyed. Although various cell types have not been characterized in dog corpora lutea, destruction of any luteal cell type could potentially result in luteolysis if cell types communicate." (citations omitted)

P. Olson *et al.*, "New Developments in Small Animal Population Control," *JAVMA*, vol. 202, pp. 904-909 (1993) gives an overview of methods for preventing or terminating unwanted pregnancies in small animals. The following discussion appears at page 905: "*Tissue-specific cytotoxins*—Permanent contraception in females and males might be achieved by administration of a cytotoxin that is linked to gonadotropin-releasing hormone (GnRH) and that selectively destroys gonadotropin-secreting pituitary cells. Similarly, a cytotoxin linked to antibodies against gonadotropin receptors could be targeted to alter gonadal function. Toxins would need to be carefully targeted to specific cells, yet be safe for all other body tissues." (citation omitted).

T. Janaky *et al.*, "Short Chain Analogs of Luteinizing Hormone-Releasing Hormone Containing Cytotoxic Moieties," *Proc. Natl. Acad. Sci. USA*, vol. 89, pp. 10203-10207 (1992) discloses the use of certain hexapeptide and heptapeptide analogs of GnRH as carriers for certain alkylating nitrogen mustards, certain anthraquinone derivatives, antimetabolite, and cisplatin-like platinum complex. The authors reported that several of the compounds exerted some cytotoxic effects on the MCF-7 breast cancer cell line.

D. Fitzgerald *et al.*, "Targeted Toxin Therapy for the Treatment of Cancer," *J. Natl. Cancer Inst.*, vol. 81, pp. 1455-1463 (1989), reviewed targeted toxin therapies for cancers, including conjugating toxins such as *Pseudomonas* exotoxin, diphtheria toxin, and ricin to a cell-binding protein such as a monoclonal antibody or a growth factor. The conjugates are then internalized into cytoplasm, where the toxin disrupts cellular activity.

Conventional targeted toxin therapies have several drawbacks. There is a small window for treatment with a particular targeted toxin (on the order of two weeks) before the recipient's immune system mounts an antibody response to the targeted toxin. These antibodies will neutralize the toxin; or worse, may result in deposition of the toxin in reticuloendothelial tissues (e.g., liver, spleen, lymph nodes, lungs, bone marrow), where they

may damage otherwise healthy tissue. Aside from this drawback, the toxin must be internalized by the targeted cell and translocated into the cytoplasm to have effect.

A related approach is to link a monoclonal antibody to an enzyme. This conjugate is directed specifically to a tumor cell surface antigen. A prodrug is then administered to the patient. The prodrug is substantially less toxic than the drug that results from activation of the drug at the tumor site by the conjugated enzyme. The activated drug then selectively attacks tumor cells. See, e.g., D. Kerr *et al.*, "Regressions and Cures of Melanoma Xenografts following Treatment with Monoclonal Antibody  $\beta$ -Lactamase Conjugates in Combination with Anticancer Prodrugs," *Cancer Research*, vol. 55, pp. 3558-3563 (1995); and H. Svensson *et al.*, "*In Vitro* and *In Vivo* Activities of a Doxorubicin Prodrug in Combination with Monoclonal Antibody  $\beta$ -Lactamase Conjugates," *Cancer Research*, vol. 55, pp. 2357-2365 (1995).

S. Sealfon *et al.*, "Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor," *Endocrine Reviews*, vol. 18, pp. 180-205 (1997) provides a review of research concerning the interaction between GnRH and its receptor.

F. Hu *et al.*, "Theophylline and Melanocyte-Stimulating Hormone Effects on Gamma-Glutamyl Transpeptidase and DOPA Reactions in Cultured Melanoma Cells," *J. Investigative Dermatology*, vol. 79, pp. 57-61 (1982) disclosed that theophylline and melanocyte-stimulating hormone (MSH) both enhanced pigmentation in murine melanoma cells, apparently by different mechanisms. J. Murphy *et al.*, "Genetic Construction, Expression, and Melanoma-Selective Cytotoxicity of a Diphtheria Toxin-Related  $\alpha$ -Melanocyte-Stimulating Hormone Fusion Peptide," *Proc. Natl. Acad. Sci. USA*, vol. 83, pp. 8258-8262 (1986) discloses selective activity against melanoma cells *in vitro* by an MSH-diphtheria toxin conjugate. See also D. Bard, "An Improved Imaging Agent for Malignant Melanoma, Based on [Nle<sup>4</sup>, D-Phe<sup>7</sup>] $\alpha$ -Melanocyte Stimulating Hormone," *Nucl. Med. Comm.*, vol. 16, pp. 860-866 (1995).

W. Siegrist *et al.*, "Homologous and Heterologous Regulation of  $\alpha$ -Melanocyte-Stimulating Hormone Receptors in Human and Mouse Melanoma Cell Lines," *Cancer Research*, vol. 54, pp. 2604-2610 (1994) reports that it is well-established that human melanoma cells possess specific high affinity receptors for  $\alpha$ -MSH. See also J. Tatro *et al.*, "Melanotropin Receptors Demonstrated In Situ in Human Melanoma," *J. Clin. Invest.*, vol. 85, pp. 1825-1832 (1990).

P. Bacha *et al.*, "Thyrotropin-Releasing Hormone-Diphtheria Toxin-related Polypeptide Conjugates," *J. Biol. Chem.*, vol. 258, pp. 1565-1570 (1983) discloses conjugates



of thyrotropin-releasing hormone (TRH) with two diphtheria toxins; one of these conjugates caused a 50% inhibition of protein synthesis in rat GH<sub>3</sub> pituitary cells at  $3 \times 10^{-9}$  M concentration. See also P. Bacha *et al.*, "Organ-Specific Binding of a Thyrotropin-Releasing Hormone-Diphtheria Toxin Complex after Intravenous Administration to Rats," *Endocrinology*, vol. 113, pp. 1072-1076 (1983).

V. Chaudhary, "Activity of a Recombinant Fusion Protein between Transforming Growth Factor Type  $\alpha$  and *Pseudomonas* toxin," *Proc. Natl. Acad. Sci. USA*, vol. 84, pp. 4538-4542 (1987) discloses that a fusion protein of a modified *Pseudomonas* toxin and transforming growth factor type  $\alpha$  selectively kills cells expressing epidermal growth factor receptors. See also D. Cawley *et al.*, "Epidermal Growth Factor-Toxin A Chain Conjugates: EGF-Ricin Is a Potent Toxin while EGF-Diphtheria Fragment A is Nontoxic," *Cell*, vol. 22, pp. 563-570 (1980).

E. Vitetta *et al.*, "Redesigning Nature's Poisons to Create Anti-Tumor Reagents," *Science*, vol. 238, pp. 1098-1104 (1987) reviews the use of immunotoxins against tumors. Uses in preventing graft-versus-host reactions are also mentioned. The authors mentioned that *in vivo* effectiveness was less than desirable. Difficulties mentioned included accessibility of toxins in circulation to target cells; instability of the linkage of toxin to antibody; rapid clearance of the immunotoxins from circulation by the liver; response by the recipient's immune system to the toxin or to the monoclonal antibody, complicating long-term therapy; possible lack of specificity for neoplastic renewal cells; cross-reactivity with normal cells; heterogeneity of tumor cells; and shedding of surface antigens by tumor cells.

P. Trail *et al.*, "Antigen-specific Activity of Carcinoma-reactive BR64-Doxorubicin Conjugates Evaluated *in Vitro* and in Human Tumor Xenograft Models," *Cancer Research*, vol. 52, pp. 5693-5700 (1992) disclose the conjugation of the anticarcinoma antibody BR64 to a doxorubicin derivative, and discuss the antitumor effects of the conjugate.

J. Olson, "Laboratory Evidence for the Hormonal Dependency of Meningiomas," *Human Reproduction*, vol. 9, supp. 1, pp. 195-201 (1994) discloses evidence that meningiomas, benign intracranial tumors, possess progesterone receptors.

S. Prigent *et al.*, "The Type 1 (EGFR-Related) Family of Growth Factor Receptors and their Ligands," *Progress in Growth Factor Research*, vol. 4, pp. 1-24 (1992) reviews the biology of the epidermal growth factor (EGF), its receptor, and related ligands and receptors (e.g., c-erbB-2, c-erbB-3, TGF $\alpha$ , amphiregulin, heregulin), and their roles in normal cell proliferation and in the pathogenesis of human cancer. See also D. Davies *et al.*, "Targeting the Epidermal Growth Factor Receptor for Therapy of Carcinomas," *Biochem. Pharm.*, vol. 51, pp. 1101-1110 (1996).

D. Morbeck *et al.*, "A Receptor Binding Site Identified in the Region 81-95 of the  $\beta$ -Subunit of Human Luteinizing Hormone (LH) and chorionic gonadotropin (hCG)," *Molecular and Cellular Endocrinology*, vol. 97, pp. 173-181 (1993) disclosed a fifteen amino acid region of LH and hCG that acted as a receptor binding site. (LH and hCG are homologous hormones that produce similar effects.)

W. Theunis *et al.*, "Luteinising Hormone, Follicle Stimulating Hormone and Gonadotropin Releasing Hormone Immunoreactivity in Two Insects: *Locusta migratoria migratoroides* R & F and *Sarcophaga bullata* (Parker)," *Invert. Reprod. and Develop.*, vol. 16, pp. 111-117 (1989) disclosed that materials immunologically related to LH, FSH, and GnRH were localized in cerebral tissue of *Locusta migratoria* and *Sarcophaga bullata*. See also P. Verhaert *et al.*, "Substances Resembling Peptides of the Vertebrate Gonadotropin System Occur in the Central Nervous System of *Periplaneta americana* L.," *Insect Biochem.*, vol. 16, pp. 191-197 (1986).

U.S. Patents No. 5,378,688; 5,488,036; and 5,492,893 disclose compounds said to be useful in inducing sterility in mammals, and in treating certain sex hormone-related cancers in mammals. The disclosed compounds were generically described as GnRH (or a GnRH analog) conjugated to a toxin. The toxin was preferably linked to the sixth amino acid of the GnRH agonist. The toxin was preferably one with a translocation domain to facilitate uptake into a cell. The inventors noted that conjugation of the GnRH agonist to the toxin "is necessary because, for the most part, the above toxins, by themselves, are not capable of binding with cell membranes in general. That is to say that applicants have found that it is only when a GnRH analog of the type described herein is linked to a toxin of the type noted above does that toxin become capable of binding to cell membranes . . . ." (E.g., Pat. No. 5,488,036, col. 7, lines 46-52.) The toxins specifically mentioned appear all to have been metabolic toxins, for example ricin, abrin, modeccin, various plant-derived ribosome-inhibiting proteins, pokeweed antiviral protein,  $\alpha$ -amanitin, diphtheria toxin, pseudomonas exotoxin, shiga toxin, melphalan, methotrexate, nitrogen mustard, doxorubicin, and daunomycin. None of these toxins is believed to be toxic due to direct interaction with the cell membrane. In the *in vivo* experiments reported, the most effective time course was reported to be weekly injections for 4 weeks. (E.g., Pat. 5,488,036, col. 20, lines 46-47.) Because most of the conjugates cited are relatively large compounds, antigenicity could be a problem when such multiple administrations are used. The GnRH analog was preferably linked to the toxin with one of several specified heterobifunctional reagents. The specifications suggest that considerable effort was expended in conjugating the toxin to the GnRH agonist. The toxins must in general be internalized into the target cells to have effect,

and do not act on cell membranes; in addition, at least some of these toxins must be secondarily transported from the membrane-bound vesicle into the cytoplasm to interact with ribosomes, mitochondria, or other cellular components.

5 M. Kovacs *et al.*, "Recovery of pituitary function after treatment with a targeted cytotoxic analog of luteinizing hormone-releasing hormone," *Proc. Natl. Acad. Sci. USA*, vol. 94, pp. 1420-1425 (1997) discloses that a doxorubin analog conjugated to an LH-RH (i.e., GnRH) agonist selectively attacked cells with LH-RH receptors, and that its effect on pituitary cells was reversible. The paper suggests that the conjugate might be used to treat tumors with LH-RH receptors. See also A. Jungwirth *et al.*, "Regression of rat Dunning R-3227-H prostate carcinoma by treatment with targeted cytotoxic analog of luteinizing hormone-releasing hormone AN-207 containing 2-pyrrolinodoxorubicin," *Intl. J. Oncol.*, vol. 10, pp. 877-884 (1997)

10 R. Moretti *et al.*, "Luteinizing hormone-releasing hormone agonists interfere with the stimulatory actions of epidermal growth factor in human prostatic cancer cell lines, LNCaP and DU 145," *J. Clin. Endocrin. & Metab.*, vol. 81, pp. 3930-3937 (1996) discloses that LH-releasing hormone agonists inhibit both androgen-dependent (LNCaP) and androgen-independent (DU 145) human prostatic cancer cell lines, and suggests that the agonists may inhibit proliferation of the tumor cells by interfering with the stimulatory actions of epidermal growth factor.

20 I. Mezô *et al.*, "Synthesis of GnRH analogs having direct antitumor and low LH-releasing activity," *J. Med. Chem.*, vol. 40, pp. 3353-3358 (1997) discloses chicken I GnRH agonists and antagonists. Agonist MI-1892 was reported to have low endocrinological activity, but to possess antitumor activity.

25 A. Nechushtan *et al.*, "Adenocarcinoma cells are targeted by the new GnRH-PE<sub>66</sub> chimeric toxin through specific gonadotropin-releasing hormone binding sites," *J. Biol. Chem.*, vol. 272, pp. 11597-11603 (1997) discloses the use of a *Pseudomonas* exotoxin coupled to GnRH to kill certain types of cancer cells.

30 X. Zhu, "Steroid-independent activation of androgen receptor in androgen-independent prostate cancer. A possible role for the MAP kinase signal transduction pathway?" *Mol. & Cell. Endocrinol.*, vol. 134, pp. 9-14 (1997) discloses that androgen receptors in prostate cancer could be activated in the absence of the androgen signal.

35 G. Emons *et al.*, "Growth-inhibitory actions of analogues of luteinizing hormone releasing hormone on tumor cells," *Trends in Endocrin. Metab.*, vol. 8, pp. 355-362 (1997) reviews the similarities and differences between GnRH receptors of cancer cells and of normal brain and pituitary cells; and suggests that LHRH analogs interfere with the mitogenic signal

transduction of growth-factor receptors and related oncogene products associated with tyrosine kinase activity in a number of malignant human tumors, including breast, ovary, endometrium, and prostate cancers.

D. Tang *et al.*, "Target to Apoptosis: A Hopeful Weapon for Prostate Cancer," *The Prostate*, vol. 32, pp. 284-293 (1997) provides a review of research on apoptosis as a route to treat prostate cancers.

A. Goustin *et al.*, "Growth Factors and Cancer," *Cancer Research*, vol. 46, pp. 1015-1029 (1986) provides an overview of various growth factors that have been associated with different cancers.

S. Cho *et al.*, "Evidence for autocrine inhibition of gonadotropin-releasing hormone (GnRH) gene transcription by GnRH in hypothalamic GT1-1 neuronal cells," *Mol. Brain Res.*, vol. 50, pp. 51-58 (1997) discloses that neuroendocrine populations of GnRH neurons have high affinity receptors for GnRH and for GnRH analogs.

S. Sower *et al.*, "Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain," *Endocrinology*, vol. 132, pp. 1125-1131 (1993) describes the structure of lamprey III GnRH.

E. Stopa *et al.*, "Immunocytochemical evidence for a lamprey-like gonadotropin-releasing hormone in human brain," *Soc. Neurosci. Abstr.*, abstract no. 437.8, p. 1577 (1987) discloses that a lamprey-like GnRH III is found in humans.

S. White *et al.*, "Three gonadotropin-releasing hormone genes in one organism suggest novel roles for an ancient peptide," *Proc. Natl. Acad. Sci. USA*, vol. 92, pp. 8363-8367 (1995); and J. Powell *et al.*, "Three forms of gonadotropin-releasing hormone characterized from brains of one species," *Proc. Natl. Acad. Sci. USA*, vol. 91, pp. 12081-12085 (1994) are examples of papers reporting the typical presence of three forms of GnRH in species of vertebrates.

J. Warnock *et al.*, "Anxiety and mood disorders associated with gonadotropin-releasing hormone agonist therapy," *Psychopharmacology Bull.*, vol. 33, pp. 311-316 (1997) reports that psychological side effects can accompany chronic treatment with a GnRH agonist.

L. Deligdisch *et al.*, "Pathological changes in gonadotropin releasing hormone agonist analogue treated uterine leiomyomata," *Fertility and Sterility*, vol. 67, pp. 837-841 reported the pathological changes associated with treating leiomyomata with a GnRH analog to induce iatrogenic menopause.

J. Fuerst *et al.*, "Effect of active immunization against luteinizing hormone-releasing hormone on the androgen-sensitive Dunning R3327-PAP and Androgen-Independent Dunning R3327-AT2.1 prostate cancer sublines," *Prostate*, vol. 32, pp. 77-84 (1997) reported that

active immunization of rats with an LHRH-diphtheria toxoid conjugate caused atrophy of the testes, prostate, and androgen-sensitive prostate tumors, with inhibition of the tumors caused by suppression of cell division rather than an increase in cell death; and that the volume increase of androgen-independent prostate tumors was slightly reduced.

5 C. Mantzoros *et al.*, "Insulin-like growth factor 1 in relation to prostate cancer and benign prostatic hyperplasia," *Br. J. Cancer*, vol. 76, pp. 1115-1118 (1997) reported that increased levels of insulin-like growth factor 1 were associated with an increased risk of prostate cancer.

10 V. Ding, "Sex hormone-binding globulin mediates prostate androgen receptor action via a novel signaling pathway," *Endocrinology*, vol. 139, pp. 213-218 (1998) reported that androgen-independent pathways may activate the progression of some prostate cancers.

J. King *et al.*, "Evolution of gonadotropin-releasing hormones," *Trends in Endocrin. Metab.*, vol. 3, pp. 339-344 (1992) discloses the primary structures of different GnRHs from various vertebrates. See also J. King *et al.*, "Structure of chicken hypothalamic luteinizing hormone-releasing hormone. II. Isolation and characterization," *J. Biol. Chem.*, vol. 257, 15 pp. 10729-10732 (1982).

N. Mores *et al.*, "Activation of LH receptors expressed in GnRH neurons stimulates cyclic AMP production and inhibits pulsatile neuropeptide release," *Endocrinology*, vol. 137, pp. 5731-5734 (1996) discloses that LH acts directly on neuroendocrine neurons in the brain. See also Z. Lei *et al.*, "Signaling and transacting factors in the transcriptional inhibition of gonadotropin releasing hormone gene by human chorionic gonadotropin in immortalized hypothalamic GT1-7 neurons," *Mol. & Cell. Endocrinology*, vol. 109, pp. 151-157 (1995). 20

United States patents 5,597,945 and 5,597,946 disclose plants transformed with genes encoding various lytic peptides. 25

#### DISCLOSURE OF INVENTION

It has been unexpectedly discovered that amphipathic lytic peptides are ideally suited to use in a ligand/cytotoxin combination to specifically inhibit abnormal or normal cells that are driven by or are dependent upon a specific ligand interaction; for example, to induce sterility or long-term contraception, or to attack tumor cells, or to selectively lyse virally-infected cells, or to attack lymphocytes responsible for autoimmune diseases. The peptides act directly on cell membranes, and need not be internalized. 30

For example, administering a combination of gonadotropin-releasing hormone (GnRH) (or a GnRH agonist) and a membrane-active lytic peptide produces long-term contraception or 35

sterilization in animals *in vivo*. Particularly surprising, sterility results even when the combination is administered to a sexually immature animal: The combination then prevents sexual maturation.

Administering *in vivo* a combination of a ligand and a membrane-active lytic peptide kills cells with a receptor for the ligand. The compounds used in the present invention are relatively small, and will not be antigenic. (Lytic peptides are known not to be very antigenic; and the ligands are not antigenic at all.) The compounds may be administered in a single dose, or in two or more closely spaced doses. Lysis of gonadotropes has been observed to be very rapid (on the order of ten minutes.) Lysis of tumor cells is rapid. The two components – the ligand and the lytic peptide – may optionally be administered as a fusion peptide, or they may be administered separately, with the ligand administered slightly before the lytic peptide, to activate cells with receptors for the ligand, and thereby make those cells susceptible to lysis by the lytic peptide. If a fusion peptide is used, it has been unexpectedly discovered that a linking moiety is not necessary to join the ligand to the lytic peptide: one may be bonded directly to the other, without the need for any intervening linkage; bonding may be performed by bonding one end of the ligand to one end of the peptide, or by bonding to the middle of either. The toxin, the lytic peptide, does not need a translocation domain, and need not be internalized, as it binds to and acts directly on the activated cell membrane to cause lysis. The ligand may be a full native compound, or it may instead be the binding domain alone; the latter is preferred where the full ligand is relatively large.

The compounds of the present invention are well-suited for use in gene therapy to treat malignant or non-malignant tumors, and other diseases caused by clones or populations of “normal” host cells bearing specific receptors (such as lymphocytes), because genes encoding a lytic peptide or encoding a lytic peptide/peptide hormone fusion may readily be inserted into hematopoietic stem cells or myeloid precursor cells.

#### MODES FOR CARRYING OUT THE INVENTION

Several cancer cells (uterine, endometrial, prostate, testicular, and ovarian) express LH or hCG receptors. Tao *et al.*, "Expression of Luteinizing Hormone/Human Chorionic Gonadotropin Receptor Gene in Benign Prostatic Hyperplasia and in Prostatic Carcinoma in Humans," *Biol. Reprod.*, vol. 56, pp. 67-72 (1997). Conjugates of a lytic peptide and LH or a portion of the LH molecule may thus be used to destroy these cells selectively. For example, the genes encoding such hormones as FSH, TRH, and LH are known, and may be linked to a DNA sequence encoding a lytic peptide to produce a secreted fusion peptide, all under the control of a suitable promoter such as the acute-phase responsive promoters

disclosed in United States patent application S.N. 08/474,678, filed June 7, 1995, and in PCT application WO 95/01095, published January 12, 1995. A binding site from a hormone may be used in lieu of the entire hormone, for example the fifteen amino acid binding site of LH and hCG. See D. Morbeck *et al.*, "A Receptor Binding Site Identified in the Region 81-95 of the  $\beta$ -Subunit of Human Luteinizing Hormone (LH) and chorionic gonadotropin (hCG)," *Molecular and Cellular Endocrinology*, vol. 97, pp. 173-181 (1993).

A powerful vector that is suitable for transforming cells to be used in gene therapy is the transposon-based vector that is disclosed in United States 5,719,055.

It is known that the D-amino acid form of GnRH will bind to gonadotropes in the pituitary, to GnRH neurons in the brain, and to various types of cancer cells. It is also known that the D-amino acid forms of lytic peptides have essentially the same propensity to lyse cell membranes as do the L-amino acid forms. Compounds of the present invention (whether administered as a fusion peptide or separately) may therefore be administered either in L-form or D-form. D-form peptides, although generally more expensive than L-form, have the advantage that they are not degraded by normal enzymatic processes, so that the D-form peptides may therefore be administered orally and generally have a longer biological half-life. Oral administration of the D-form peptide may be enhanced by linking the peptide/hormone fusion product to a suitable carrier to facilitate uptake by the intestine, for example vitamin B<sub>12</sub>, following generally the B<sub>12</sub>-conjugation technique of G. Russell-Jones *et al.*, "Synthesis of LHRH Antagonists Suitable for Oral Administration via the Vitamin B<sub>12</sub> Uptake System," *Bioconjugate Chem.*, vol. 6, pp. 34-42 (1995).

GnRH or GnRH analogs (collectively, "GnRH agonists") may be used in the present invention. It has been reported that substitutions at the 6 and 10 positions of the GnRH decapeptide can produce "superagonists" having greater binding affinity to the GnRH receptor than does GnRH itself. These "superagonists" include goserelin, leuprolide, buserelin, and nafarelin. See U.S. Patent 5,488,036.

Without wishing to be bound by this theory, it is believed that a mechanism (though not the exclusive mechanism) underlying the sterilization/long term contraception aspect of this invention is as follows: GnRH activates gonadotropic cells in the pituitary gland, as well as neuroendocrine GnRH neurons in the brain. The activated cells have substantially increased susceptibility to lysis by a lytic peptide. The lytic peptide then preferentially destroys (or severely damages) these activated cells. When the gonadotrophic cells in the pituitary are destroyed and are deprived of GnRH from the brain, the pituitary no longer

secretes follicle stimulating hormone (FSH) or luteinizing hormone (LH), rendering the animal temporarily or permanently sterile.

Although the ligand and the lytic peptide may be administered separately, it is preferred to link the two in a single molecule, because such a linkage greatly increases the effective concentration of the lytic peptide in the vicinity of ligand-activated cells. Furthermore, this increase in the effective lytic peptide concentration can obviate the need for activation of the cells, allowing the peptide to be linked to a binding site of a ligand alone, without needing to include the "remainder" of a native ligand that would normally be needed for activating the target cells. This linkage may be in either order: for example, GnRH/peptide or peptide/GnRH. Examples are modified GnRH/hecate (SEQ. ID NO. 3) and hecate/modified GnRH (SEQ. ID NO. 4). Note that no intermediate linker is necessary, and that the carboxy terminus of one of the two peptides may be bonded directly to the amino terminus of the other. (We have found that the initial pyro-glutamic acid residue of GnRH or of the GnRH portion of a fusion peptide may be substituted with glutamine without substantially changing the activity of the respective peptides. See, e.g., SEQ. ID Nos. 9, 3, and 4.)

### ***Experimental Results***

#### **Examples 1-6**

The pituitary gland of an adult female rat was harvested and divided into six sections of approximately equal size. One section was placed in each of six wells containing tissue culture medium at 37°C. A different treatment was applied to each well, as described below. Ten hours after treatment, the tissue from each well was fixed, and the histology of each was examined microscopically.

**Treatment 1** applied tissue culture medium alone as a control. The histology of this tissue after treatment appeared normal.

**Treatment 2** was an application of 5 nanograms of GnRH (SEQ. ID NO. 1) per mL of medium. The histology of this tissue after treatment was normal; a small degree of cellular vacuolization was noted. For comparison, the concentration of GnRH in normal, untreated rats varies from as low as 1 ng/mL to as high as 20 ng/mL during the LH surge phase of the estrous cycle.

**Treatment 3** was an application of 50  $\mu$ M of the lytic peptide hecate (SEQ. ID NO. 2) in the medium. The histology of this tissue after treatment appeared normal.

**Treatment 4** was an initial application of 5 nanograms of GnRH per mL of medium for 15 minutes. Following this incubation, the medium containing GnRH was removed, and



the tissue was washed once with plain medium. This medium was then removed, and was replaced with medium containing 50  $\mu$ M of the lytic peptide hecate. Widespread basophilic (gonadotropic) cellular destruction was observed after this treatment.

**Treatment 5** was an application of 50  $\mu$ M of the fusion peptide modified GnRH/hecate (SEQ. ID NO. 3). Widespread basophilic (gonadotropic) cellular destruction was observed after the treatment.

**Treatment 6** was an initial application of the fusion peptide GnRH/hecate (SEQ. ID NO. 3), followed by a second application of the fusion peptide GnRH/hecate two hours later. After treatment the tissue was virtually destroyed, with only stromal cells remaining.

#### **Example 7**

Two sexually immature female rats from the same litter (age 33 days) were given two intravenous injections of saline control solution 24 hours apart. After the rats reached breeding age, they were examined 105 days post-inoculation. The external genitalia appeared normal. During a fourteen-day monitoring period 107 days to 121 days post-inoculation, each of the control rats completed at least two estrous cycles. The rats were then sacrificed and necropsied. The reproductive organs appeared histologically normal.

#### **Example 8**

Two sexually immature female rats from the same litter as those of Example 7 (age 33 days) were given two intravenous injections of 500  $\mu$ g GnRH/hecate fusion peptide in saline 24 hours apart. After the rats reached breeding age, they were examined 105 days post-inoculation. The external genitalia appeared small. Unlike the control rats, insertion of a cotton-tipped swab into the vagina was difficult. During a fourteen-day monitoring period 107 days to 121 days post-inoculation, neither of the treated rats demonstrated estrous or metestrous. The rats were then sacrificed and necropsied. The peptide-treated rats had thinned, inactive uterine and oviductal epithelia. Their ovaries contained no large follicles, and had a high number of atretic follicles (i.e., those that had failed to ovulate).

#### **Examples 9-14**

Eighteen sexually mature, mixed breed, female rats were randomly assigned to one of six groups containing three rats each. Each group of rats received a double treatment intravenously, as described below. Two weeks after the treatment, the rats were sacrificed and necropsied. The reproductive and endocrine organs were sectioned, weighed, and examined histologically.

**Treatment 9** was a saline control. The rats in this group exhibited normal ovarian function (e.g., normal follicles and new corpora lutea). The pituitaries from this group were of normal size. Histology showed a normal number of pituitary basophilic cells.

**Treatment 10** was injection with a total of 1.0 mg GnRH/hecate fusion peptide in saline, divided into two equal 0.5 mg injections administered 24 hours apart. The rats in this group showed an arrest of normal ovarian follicular development. Few corpora lutea were present, and those that were present appeared old. Follicles were large, and had not ruptured. Uterine morphology was consistent with hormonal inactivity. The pituitaries from this group were slightly smaller than the pituitaries from the saline control group. Histology revealed a 60% to 70% reduction in the number of pituitary basophilic cells compared to the controls.

**Treatment 11** was injection of 100  $\mu$ L of a 1.35 mM solution of GnRH (162  $\mu$ g) in saline, followed 15 minutes later by injection with 100  $\mu$ L of a 1.35 mM solution of hecate (337  $\mu$ g) in saline. The same two-step treatment was repeated 24 hours later. The rats in this group showed altered ovarian histology. Few corpora lutea were present, and those that were present appeared old. Follicles were large, and had not ruptured. Uterine morphology was consistent with hormonal inactivity. The pituitaries and the pituitary histology were similar to those observed in Treatment 10.

**Treatment 12** was injection of 100  $\mu$ L of a 1.35 mM solution of hecate (337  $\mu$ g) in saline. The treatment was repeated after 24 hours. The rats in this group exhibited normal ovarian function (e.g., normal follicles and new corpora lutea). The pituitaries and the pituitary histology were similar to those observed in Treatment 9.

**Treatment 13** was injection of 100  $\mu$ L of a 1.35 mM solution of GnRH (162  $\mu$ g) in saline. The treatment was repeated after 24 hours. The rats in this group exhibited normal ovarian function (e.g., normal follicles and new corpora lutea). The pituitaries and the pituitary histology were similar to those observed in Treatment 9.

**Treatment 14** was identical to Treatment 10, except that the GnRH/hecate fusion peptide was further purified by HPLC. The rats in this group showed an arrest of normal ovarian follicular development. Few corpora lutea were present, and those that were present appeared old. Follicles were large, and had not ruptured. Uterine morphology was consistent with hormonal inactivity. The pituitaries and the pituitary histology were similar to those observed in Treatment 10.

These experiments demonstrate that GnRH and the lytic peptide may be administered either separately or as a fusion peptide, although the fusion peptide is preferred as it is expected to be more active at lower doses.

Although experiments to determine optimum dosages had not been performed by the time this application is being filed, a person of ordinary skill in the art, who is given the teachings of the present specification, may readily ascertain optimum dosages through routine testing.

Although the experiments to date have been performed on female animals, similar results are expected for male animals, because GnRH signals pituitary cells to release gonadotropins in both males and females.

Tissue and cell specificity of cytotoxic conjugates could be further enhanced by using various hormones or hormone analogs coupled to a lytic peptide. Some examples follow. For fertility control, both the pituitary and the central GnRH neuronal component of the reproductive axis are selectively damaged by GnRH-hecate conjugate. Few cells in the central nervous system should be damaged by this treatment, because the chicken II GnRH and lamprey III GnRH forms are the primary molecules affecting brain function in most mammals. Fertility control may also be selectively accomplished by treating animals with a bLH-hecate conjugate; this compound should specifically affect GnRH neurons controlling reproduction and the gonads. To target prostatic, breast, ovarian, or endometrial cancer cells, the l-LHRH-III-hecate conjugate could be used since it binds to receptors on cancer cells, and has no significant known action on the brain. (Other lytic peptides may be used in place of hecate in these conjugates.)

The compositions of the present invention may be administered as described, or as pharmaceutically acceptable salts. The compositions may be administered intravenously, subcutaneously, intramuscularly, or orally (especially when in D-amino acid form, preferably complexed with a carrier, e.g., vitamin B<sub>12</sub>).

Applications of the present invention include long-term contraception or sterilization in humans; and long-term contraception or sterilization in domesticated or wild mammals, birds, reptiles, amphibians, bony fish, cartilaginous fish, jawless fish, and invertebrates such as insects or molluscs. Domesticated mammals in which this invention may be used include, for example, dogs, cats, cattle, horses, pigs, and sheep. When used in humans, long-term replacement hormone therapy may be needed to prevent undesirable side effects, such as premature menopause. Administration of gonadotropic hormones in a sterilized individual will temporarily restore fertility if desired. The sterilization is reversible in this sense.

As one example, this invention may be used in the humane population control of an unwanted introduced species.

5 Sterilization of domesticated birds such as chickens and turkeys can increase their growth rate. Avian GnRH or analogs may be used in practicing this invention to sterilize birds. There are two forms of avian GnRH -- Chicken I GnRH (SEQ. ID NO. 17) and  
10 Chicken II GnRH (SEQ. ID NO. 18). Either form of avian GnRH may be used in this invention. In a preferred embodiment, position 6 of Chicken I GnRH is linked to a lytic peptide such as hecate to form a fusion peptide. Alternatively, a GnRH agonist or antagonist may be used. A series of agonists and antagonists has been synthesized by I. Mezo *et al.*,  
15 "Synthesis of GnRH analogs having direct antitumor and low LH-releasing activity," *Biomed. Peptides, Proteins & Nucleic Acids*, vol. 2, pp. 33-40 (1996).

When used to treat insects that are pests to crop plants or other plants, it may be desirable to incorporate genes encoding the peptide/ligand combination into the plant's genome, under the control of a promoter that expresses the peptide in tissues of the plant that  
15 are attacked by the insect, but not in tissues that are used for food. For example, in a potato a promoter could be used that is active in the leaves of the plant, but not in the tuber. Expression in the plant tissue could be constitutive, or alternatively could be induced by stimuli that induce the plant's native defense mechanisms, for example by placing the peptide gene under the control of native promoters that are so induced in plants. See, e.g., United  
20 States patent application S.N. 08/279,472, filed July 22, 1994.

When used to sterilize aquatic animals such as fish or molluscs, the compounds of the present invention may be simply administered in the water, from which they will be taken up by the animals in adult, juvenile, or larval stages. Preferably, the peptides are encapsulated in liposomes, which are fed to the animals as spat, fry, juveniles, or adults; the animals feed on  
25 the liposomes, which then release the compounds into the animal's circulation, causing sterilization. Alternatively, the peptides may be injected into an animal that has reached sufficient size.

For example, the compounds may be used to sterilize undesirable exotic molluscs such as the zebra mussel. Sterilization of aquaculture species may also be desirable. For example,  
30 sterilization of oysters will prevent the oysters from ripening gonads in the summer (when they would otherwise do so), thereby improving their marketability.

#### Examples 15-22

Eight sexually mature, Sprague-Dawley female rats were randomly assigned to one of  
35 eight treatments. Each group of rats received a single treatment intravenously, as described

below. Rats were sacrificed and necropsied either 48 or 96 hours after treatment. The ovaries, uterus, pancreas, liver, spleen, lungs, heart, thyroid, and adrenal glands were fixed in 10% buffered formalin; sectioned; and stained with H&E (hematoxylin and eosin) stain; except that the pituitary glands were stained with PAS (periodic acid-Schiff) stain with no counter-stain. The treatments were selected so that each animal received an equimolar amount of the compound with which it was treated.

**Treatments 15 and 16** were IV-injection with 674  $\mu$ g of D-hecate in 200  $\mu$ L saline (1.35 mM). The rat in treatment 15 was sacrificed 48 hours after injection, and the rat in treatment 16 was sacrificed 96 hours after injection. No gross lesions were noted in the organs of either animal. The pituitary glands of both rats contained a normal number of PAS-positive cells.

**Treatments 17 and 18** were IV-injection with 334  $\mu$ g of GnRH in 200  $\mu$ L saline (1.35 mM). The rat in treatment 17 was sacrificed 48 hours after injection, and the rat in treatment 18 was sacrificed 96 hours after injection. No gross lesions were noted in the organs of either animal. The pituitary glands of both rats contained a normal number of PAS-positive cells.

**Treatments 19-22** were IV-injection with 1 mg GnRH-hecate fusion peptide (SEQ. ID NO. 3) in 100  $\mu$ L saline (2.7 mM). The rats in treatments 19 and 20 were sacrificed 48 hours after injection, and the rats in treatments 21 and 22 were sacrificed 96 hours after injection. No gross lesions were noted in the organs of any of the four animals, other than the pituitary. The pituitary glands of the animals from treatments 19 and 20 were slightly enlarged, hyperemic, and edematous. The pituitary glands of the animals from treatments 21 and 22 were slightly hyperemic, but of expected size. The pituitary glands of all four rats showed a marked depletion of PAS-positive cells; it was estimated that the depletion was 80 to 90% compared to those of control groups. (PAS stain preferentially stains glycopeptides. LH, FSH, TSH, and MSH are glycopeptide hormones; cells containing these hormones stored in their secretory vacuoles stain positive with PAS.)

It was thus seen that the GnRH-lytic peptide combination caused morphological and functional alterations in the adult female rat reproductive system, and in preventing sexual maturity in pre-pubertal female rats, but that the fusion peptide selectively eliminated a specific population of PAS-positive staining cells in the pituitary.

**Example 23**

Hecate is an amphipathic lytic peptide that acts on cell membranes without being internalized. It is a synthetic peptide analog of melittin, the primary toxin in honeybee venom. Hecate is believed to act by disrupting cell membranes. The structure of the modified GnRH-hecate conjugate used in these studies was SEQ. ID NO. 3.

We also synthesized D-Lys<sup>6</sup>GnRH (SEQ. ID NO. 13), so that hecate could be conjugated to the D-Lys<sup>6</sup>, a position that could minimize interference with binding of the GnRH domain to the GnRH receptor. These synthetic peptides specifically displaced radiolabelled monoiodinated-GnRH from rat pituitary membranes. Displacement by D-Lys<sup>6</sup>GnRH-hecate was comparable to (and actually slightly greater than) displacement by native mammalian GnRH, as measured by cpm of radioactivity. When GnRH and GnRH-hecate binding were compared on a molar basis over a 1000-fold concentration range ( $n = 6$ ) the GnRH-hecate specifically displaced the radiolabelled peptide to an extent equal to  $123\% \pm 4\%$  of the binding exhibited by equimolar concentrations of GnRH; equimolar concentrations of D-Lys<sup>6</sup>GnRH displaced  $187\% \pm 8\%$  of the cpm displaced by native GnRH.

**Examples 24-31**

We studied *in vitro* lysis of bovine luteal cells with GnRH-hecate conjugate and with hecate-bLH conjugate (SEQ. ID NO. 12). (The bLH component of the conjugate is a 15-mer fragment of the beta chain of luteinizing hormone, SEQ. ID NO. 11). Small luteal cells were collected from cattle corpora lutea post-slaughter. Small luteal cells are rich in LH receptors, and were found to be highly susceptible to lysis by the hecate-bLH conjugate.

Small luteal cells in culture were incubated with one of the following treatments for 22 hours, and were then examined for viability using Trypan Blue exclusion and release of lactic dehydrogenase.

**Treatment 24** control: no additional treatment (media alone)

**Treatment 25** 10 ng bLH (positive control)

**Treatment 26** hecate-bLH, 10  $\mu$ M

**Treatment 27** hecate-bLH, 5  $\mu$ M

**Treatment 28** hecate-bLH, 1  $\mu$ M

**Treatment 29** hecate (alone), 10  $\mu$ M

**Treatment 30** hecate (alone), 5  $\mu$ M

**Treatment 31** hecate (alone), 1  $\mu$ M

Significant killing of small luteal cells was observed following 22 hr. incubation with 10  $\mu$ M hecate alone, and with 5  $\mu$ M hecate alone (approximately 50% killing). Cell death for 1  $\mu$ M hecate alone did not differ significantly from negative control (media) or from bLH alone. All three treatment doses with hecate-bLH caused significant increases in cell death as compared to treatment with hecate alone. The hecate-bLH conjugate killed approximately twice the number of cells as were killed by hecate alone at the same concentrations.

Observed levels of lactic dehydrogenase activity also demonstrated that the hecate-bLH treatment killed a significantly greater number of cells than did hecate alone.

### Examples 32-33

We also studied *in vitro* lysis of bovine granulosa cells with GnRH-hecate conjugate and with hecate-bLH conjugate. Granulosa cells were isolated from bovine pre-ovulatory follicles. (Granulosa cells are hormonally active cells with numerous LH receptors.) Our experiments with granulosa cells were otherwise generally similar to those described above for Examples 24-31. These experiments demonstrated (1) that the granulosa cells were much more susceptible to killing by hecate alone than were the small luteal cells, and (2) that, as had been the case with the small luteal cells, the granulosa cells were significantly more susceptible to hecate-bLH at even the lowest concentration (1  $\mu$ M) than they were to hecate alone. At 1  $\mu$ M, the hecate-bLH conjugate killed about twice the number of target cells as did hecate alone. Again, the levels of lactic dehydrogenase released following the hecate-bLH 1  $\mu$ M treatment were significantly higher than the levels of enzyme released following treatment with 1  $\mu$ M hecate alone.

Additional studies (data not shown) demonstrated that a 15-mer fragment of the bLH subunit specifically bound to LH receptors on the target granulosa cells, but did not initiate the production of steroid hormones that would be indicative of a stimulus-coupled response. We thus demonstrated that the selective killing of target cells resulted from the physical proximity of the lytic peptide to the cell, which was caused by binding of the LH subunit. Stimulation of target cell hormone production was not required for cell lysis. This result was surprising, as we had previously expected that activation of the target cells was required for increased susceptibility to lysis. These data demonstrate that such activation is not required. These data are, however, consistent with our other data showing that cell activation is also a route that can lead to increased susceptibility to the lytic peptide.

### Examples 34-37

Another set of experiments was performed to study the *in vivo* effects of the GnRH-hecate conjugate on female rats and rabbits. The ovaries, uterus, oviducts, adrenals, spleen,

thyroids, pancreas, liver, lungs, and heart were processed for histological analysis. The pituitaries were processed for histological analysis of PAS-stained cells and for cells stained immunocytochemically for bLH, BFSH (bovine follicle stimulating hormone), adrenocorticotrophic hormone, and other proopiomelanocortin peptide products (most notably alpha-melanocyte stimulating hormone (MSH)), thyroid stimulating hormone (TSH), prolactin (PRL), vasopressin (VP), oxytocin (OXY) or growth hormone (GH). The immunocytochemical staining procedures we used followed generally the procedures of M. Rahmanian *et al.*, "Histological and immunocytochemical characterization of pituitary cell types in ponies," *Proc. 13th Soc. Equine Nutrition & Phys. Symp.*, pp. 348-349 (1993); M. Rahmanian *et al.*, "Immunocytochemical localization of luteinizing hormone and follicle-stimulating hormone in the equine pituitary," *J. Anim. Sci.*, vol. 76, pp. 839-846 (1998); M. Rahmanian *et al.*, "Immunocytochemical localization of prolactin and growth hormone in the equine pituitary," *Animal Sci.*, vol. 75, pp. 3010-3018 (1997); and P. Melrose *et al.*, "Comparative topography of the immunoreactive alpha-melanocyte-stimulating hormone neuronal system in the brains of horses and rats," *Brain Beh. & Evol.*, vol. 32, pp. 226-235 (1988).

Brains were serially sectioned on a Vibrotome from the level of the diagonal band of Broca to the mammillary body. Alternate sections were consecutively divided into four to five dishes, and sections in alternate dishes were stained with cresyl violet, or were stained immunocytochemically for GnRH or the GnRH precursor, VP, OXY, or tyrosine hydroxylase (the rate-limiting enzyme in catecholamine synthesis). In addition to the staining procedures cited above, we also used the immunocytochemical staining procedures of P. Melrose *et al.*, "Distribution and morphology of immunoreactive gonadotropin-releasing hormone (GnRH) neurons in the basal forebrain of ponies," *J. Comp. Neurol.* vol. 339, pp. 269-287 (1994); and P. Melrose *et al.*, "Topography of oxytocin and vasopressin neurons in the forebrain of *Equus caballus*: Further support of proposed evolutionary relationships for proopiomelanocortin, oxytocin and vasopressin neurons," *Brain, Beh. & Evol.*, vol. 33, pp. 193-204 (1989).

Thirty-three-day-old, sexually immature female rats were given intravenous administrations as follow:

- Treatment 34:** 0.03  $\mu$ g GnRH (a normal physiological dose) (two rats)
- Treatment 35:** 1.62  $\mu$ g GnRH (the molar equivalent to the amount of GnRH in Treatment 36) (one rat)
- Treatment 36:** 0.5 mg GnRH-hecate (one rat)



**Treatment 37:** 0.03  $\mu$ g GnRH, followed 11 minutes later by 0.337  $\mu$ g hecate (two rats).

Animals were sacrificed 14 days after treatment. As compared to the two GnRH control groups, the treatment with GnRH-hecate and the treatment with GnRH followed by hecate alone reduced pituitary weights by 13% and 14%, respectively, and reduced the numbers of bLH-specific gonadotropes by 92% and 87%, respectively. Further, following these two experimental treatments the cell bodies of GnRH-stained neurons in hypophysiotropic areas of the brain were frequently deformed; and a substantial amount of immunoreactive material leached into surrounding areas where numerous cell bodies are concentrated (the organum vasculosum of the lamina terminalis). There was histological damage to cells from the two experimental treatments in the C1 and C3 fields of the hippocampus, and increased staining of parvicellular VP neurons in the paraventricular nucleus. (The VP staining may have been caused by formation of a precipitate in certain areas of the brain. Subsequent studies with more highly purified peptide did not show a precipitate). The change in VP expression, probably in corticotropin-releasing neurons, may cause a shift in the post-translational processing of proopiomelanocortin peptide products in the pars distalis, since GnRH-hecate and GnRH + hecate treatments reduced adrenocorticotrophic hormone levels and increased the number of alpha-MSH-stained cells in this subdivision of the pituitary. No pathological changes were noted in any other tissues.

Since neurons in the brain do not regenerate, severe damage to these neurons could make sterilization with a GnRH/lytic peptide combination permanent (but temporarily reversible by administration of gonadotrophic hormones).

#### **Examples 38-42**

Sexually immature (33 day old) female rats (randomly allocated into groups of three) were injected intravenously with saline or GnRH-hecate in saline as follows:

**Treatment 38:** 0.0 mg GnRH-hecate

**Treatment 39:** 0.1 mg GnRH-hecate

**Treatment 40:** 0.5 mg GnRH-hecate

**Treatment 41:** 1.0 mg GnRH-hecate

**Treatment 42:** 1.5 mg GnRH-hecate.

Animals were sacrificed at 24 hours or at 14 days after treatment. Results were similar to those reported above for Examples 34-37, except that no precipitate was found in the brain, and VP staining in the CNS was not altered. The treatments with higher levels of GnRH-hecate produced a large number of GnRH-receptor-containing neurons having abnormal

morphologies, including distortion of the somatic portion of the cells, and degeneration of neurites. In the rats sacrificed fourteen days after treatment, 66% and 87% of the GnRH-receptor-containing neurons were abnormal in the rats that had received 1.0 and 1.5 mg of GnRH-hecate, respectively. Axonal degeneration in the 1.5 mg GnRH-hecate group was accompanied by over 90% reduction in median eminence staining for GnRH.

#### Examples 43-45

Seven sexually mature female New Zealand rabbits were injected intravenously with saline containing GnRH-hecate as follows:

**Treatment 43:** 0 mg GnRH-hecate (n = 3)

**Treatment 44:** 5 mg GnRH-hecate (n = 3)

**Treatment 45:** 10 mg GnRH-hecate (n = 1).

Forty-six days later all rabbits were injected intramuscularly with 100 µg GnRH. Blood samples were collected at 0, 1, 4, and 24 hours, and LH and FSH levels in the blood samples were measured by radioimmunoassay. Hormone analyses revealed that both control and experimental animals released LH in response to the GnRH, suggesting that there may be at least some degree of reversibility following treatment, at least for pituitary gonadotropes at lower doses of ligand/peptide. The rabbits were sacrificed the next day (day 47) for postmortem histological analysis. We found that the numbers of tertiary follicles, corpora lutea, and GnRH-induced ovulations were reduced by GnRH-hecate treatment. Ovarian and pituitary weights were reduced by the 10 mg GnRH-hecate treatment. In tissues from the GnRH-hecate treatments, observed immunoreactive GnRH was faint and diffusely localized in CNS areas normally containing cell bodies; normal individual cell bodies were reduced in number by at least 50%; and the terminal fields, which normally contain the axons of GnRH receptor neurons, were not stained for GnRH. These observations suggest that the most pronounced effects of the GnRH-hecate treatments in these experiments on rabbits may have been on neuroendocrine neurons in the brain. The hippocampus and other areas of the brain containing high concentrations of GnRH were not discernibly affected by GnRH-hecate treatments. The GnRH-hecate treatment increased the number of PAS-stained pituitary cells in the pars distalis to 177% of that for control rabbits; this increase appeared to reflect increased numbers of cells staining alpha-MSH, and reduced numbers of cells staining for LH.

#### Examples 46-47

Nine sexually mature female rabbits were injected intravenously with saline containing 0 mg (n = 4) (Treatment 46) or 10 mg GnRH-hecate (n = 5) (Treatment 47). Rabbits were injected intramuscularly with GnRH on day 6 posttreatment. Blood samples were collected

for radioimmunoassay of LH and FSH as described above, and the animals were sacrificed on day 7 post-treatment. Both control and experimental animals released LH in response to the GnRH; however, the amount of LH released was lower in the treated animals than in the controls. The GnRH-hecate treatment reduced the numbers of tertiary ovarian follicles, and the numbers of GnRH-induced ovulations. No effects were noticed either on peripheral tissues or on pituitary weight. The effects of GnRH-hecate on CNS morphology and immunocytochemical results were similar to those described above in Examples 34-45. Again, the effects were more pronounced on GnRH neurons than on staining of pituitary gonadotropes.

The number of ovulation sites in rabbits in Examples 46 and 47 treated with 10 mg GnRH-hecate were reduced as compared to saline controls. The mean number of ovulation sites in four saline controls equalled  $12.2 \pm 5.4$ , with S.E.M. = 2.7. The mean number of ovulation sites in the five rabbits given 10 mg of GnRH-hecate was  $3.6 \pm 1.1$ , with S.E.M. = 0.5. This difference from control was significant ( $p = 0.025$ ).

The "LH surge" (the level of LH at one hour post-GnRH challenge, minus the resting level before challenge) in the four controls was  $61.2 \pm 16.5$  ng/mL, with S.E.M. = 8.3; and in the treated group was  $49.6 \pm 26.1$  ng/mL, with S.E.M. = 12 ( $p = 0.22$ ). Thus there was a trend towards lower LH levels in the treated group.

The *in vivo* studies clearly demonstrated that the GnRH-hecate conjugate selectively damaged GnRH receptor-bearing cells in the brain (neurons) and in the pituitary (gonadotrophic cells). Further, these studies demonstrated a significant alteration in the ovary, presumably a consequence of alteration in the reproductive centers of the brain-pituitary axis. Selectivity of the conjugate was demonstrated by the following observations: (1) No cytotoxic changes were seen in neurons that lacked GnRH receptors. (2) No changes were seen in pituitary cells that lacked GnRH receptors. (3) No changes were seen in other endocrine and non-endocrine tissues (except for the ovary, which presumably responded indirectly to the destruction of gonadotrophs in the pituitary).

Many of the events referred to as "ovulations" in the GnRH-hecate treated rabbits possibly were not functional ovulation sites, but may instead have represented hemorrhagic pre-ovulatory degenerative changes. Additional breeding trials will be conducted to verify that ovulation of functional ova is prevented.

**Examples 48-51**

The following examples demonstrated the ability of a GnRH-lytic peptide combination to reduce fertility in insects. It was also unexpectedly discovered that the lytic peptide alone (i.e., administered without GnRH) had similar effects. Although insects are not believed to secrete a GnRH identical to that found in mammals, there appears to be some homology, in that the insects did respond to mammalian GnRH, and to GnRH linked to the lytic peptide hecate.

Late-stage *Diatraea saccharalis* (sugar cane borer) pupae were inoculated with 1.0  $\mu$ L of saline solution containing 1.35 mM concentration of peptide as stated, or saline alone as control. The pupae were allowed to complete metamorphosis. No gross morphological defects were observed in any of the insects completing metamorphosis. Adult female moths were allowed to mate with treated males, and then lay eggs. The viability of the eggs was measured by counting the number hatching into larvae.

**Treatment 48** was the control, inoculation of 21 pupae with saline alone. Twelve of the pupae completed metamorphosis into adult moths (4 males, 8 females). The 8 females laid a total of about 900 eggs, an average of about 112 eggs per female. About 22% of these eggs hatched, or about 25 hatched larvae per female.

**Treatment 49** was inoculation of 10 pupae with 1.35 mM GnRH. Four of the pupae completed metamorphosis into adult moths (2 males, 2 females). The 2 females laid a total of about 300 eggs, or an average of about 150 eggs per female. About 40% of these eggs hatched, or about 60 hatched larvae per female.

**Treatment 50** was inoculation of 10 pupae with 1.35 mM GnRH-hecate (SEQ. ID NO. 3). Eight of the pupae completed metamorphosis into adult moths (3 males, 5 females). The 5 females laid a total of about 200 eggs, or an average of about 40 eggs per female. About 40% of these eggs hatched, or about 16 hatched larvae per female.

**Treatment 51** was inoculation of 10 pupae with D-hecate. Six of the pupae completed metamorphosis into adult moths (2 males, 4 females). The 4 females laid a total of 18 eggs, or an average of 4.5 eggs per female. 100% of these eggs hatched, or 4.5 hatched larvae per female.

It was thus observed that, compared to controls, females treated with GnRH alone in the late pupal stage had enhanced reproductive success; those treated with the GnRH-hecate combination had decreased reproductive success; and those treated with D-hecate alone had even lower reproductive success.

Without wishing to be bound by the following hypothesis, it is believed that these results may be explained as follows. Due to (as yet unidentified) sequence homology across taxa, small peptides active in the control of mammalian reproduction also influence reproductive function in insects. See W. Theunis *et al.*, "Luteinising Hormone, Follicle Stimulating Hormone and Gonadotropin Releasing Hormone Immunoreactivity in Two Insects: *Locusta migratoria migratoroides* R & F and *Sarcophaga bullata* (Parker)," *Invert. Reprod. and Develop.*, vol. 16, pp. 111-117 (1989); and P. Verhaert *et al.*, "Substances Resembling Peptides of the Vertebrate Gonadotropin System Occur in the Central Nervous System of *Periplaneta americana* L.," *Insect Biochem.*, vol. 16, pp. 191-197 (1986).

This activity is probably mediated by the inherent ability of these peptides to react with appropriate intermediate cells by a ligand-receptor interaction, thus altering the functional activity of the intermediate cells. More particularly, insects have a receptor that responds to mammalian GnRH. GnRH alone stimulates reproductive activity in insects. GnRH coupled to a lytic peptide attacks the intermediate cells in the insects, inhibiting reproductive activity.

The results observed for the D-hecate administered without GnRH were surprising, and are explained somewhat differently, again without wishing to be bound by the following hypothesis. Metamorphosis is a time of high cell activity. Lytic peptides generally have greater activity against active cells. The observed response to hecate alone is believed to be a generalized response by activated cells, not a specific response mediated by a receptor. The fact that the D-conformation of hecate was used in this experiment may be significant, since D-form peptides generally have a longer biological half-life. It is currently unknown whether similar results would be seen with L-hecate alone. (D-hecate was used in Treatment 26 for the simple reason that previously-synthesized D-hecate was readily available to the investigators.)

#### ***Treatment of Malignant and Benign Tumors***

The compositions of the present invention are useful in killing or inhibiting the growth of malignant and benign tumor cells that express receptors for GnRH, LH, hCG, I-LHRH-III, or steroids. The ligand is administered with a lytic peptide (either sequentially, or linked to one another), and the targeted tumor cells are killed or inhibited.

In treating hormone- or ligand-linked cancers (e.g., cancers of the ovary, testis, breast, uterus, endometrium, pituitary, and prostate), lytic peptides may be attached to the hormone for which the tumor expresses a receptor or set of receptors, e.g., an estrogen, testosterone, LH, FSH, estradiol-17 $\beta$ , transforming growth factor alpha (TGF $\alpha$ ), epidermal growth factor (EGF), GnRH, LH, hCG, lamprey III LHRH (I-LHRH-III), and melanocyte stimulating hormone. For example, an ester linkage of a lytic peptide to estradiol or testosterone can conveniently be made by condensing the carboxy terminus of the lytic peptide with the hydroxyl group at the 17-carbon position of the steroid. An estradiol/lytic peptide combination may be used as a treatment against breast or ovarian cancer; and a testosterone/lytic peptide combination may be used to treat prostate cancer. In addition, the specific binding domains of the peptide hormone LH or FSH may be used in fusion peptides with a lytic peptide to selectively bind the fusion peptide to target tumor cells with cell surface receptors for these hormones. For example, the receptor binding site of the  $\beta$ -subunit of LH and hCG may be used (SEQ. ID NO. 11). See Morbeck *et al.*, *Mol. and Cell Endocrin.*, vol. 97, pp. 173-186 (1993).

#### ***Pituitary Tumors***

The anterior pituitary contains different types of epithelial cells that control the complex processes of growth, reproduction, lactation, thyroid function, and adrenal functions. Due to the high functional plasticity of pituitary cells (i.e., their ability to differentiate into different cellular phenotypes in response to stimuli), these cells are particularly prone to aberrant behavior. Because many of the signals to which the pituitary responds are receptor-mediated, pathological states may be controlled by co-opting the appropriate ligand-receptor interaction. Several examples are given below.

#### ***Dopamine Receptors in Prolactinomas and other Adenomas***

Chronic dopamine deficiency has been associated with some types of pituitary tumors. In certain adenomas the number of dopamine binding sites is reduced by about 50%, and the number can be reduced even further during dopaminergic therapy. It has also been reported that nerve growth factor can stimulate prolactinoma cells to re-express dopamine receptors. Pretreating a prolactinoma with nerve growth factor before treatment with a dopamine/lytic peptide combination makes it susceptible to treatment through the present invention. The lytic peptide may be linked to dopamine, for example, by an amide group formed by condensing the carboxy terminus of the peptide with the amino group of dopamine.

This therapy will be effective not only for prolactinomas, but also for other adenomas expressing dopamine receptors, such as growth hormone-secreting adenomas, thyrotropin-releasing hormone secreting adenomas, and gonadotropin-secreting adenomas.

#### ***Somatostatin Receptors in Growth Hormone-Secreting Adenomas***

It has been reported that growth hormone (GH)-secreting adenomas have a highly variable number of somatostatin receptors. (Variation by at least a factor of 10 may be seen among individual tumors.) There is also considerable variation in the distribution of binding sites: the somatostatin receptors may be homogeneously distributed, located exclusively in one portion of the tumor tissue, or in between.

Somatostatin receptors are also present in other types of pituitary tumors. It has been reported that the cell surfaces of a majority of GH- and thyrotropin releasing hormone (TRH)-secreting adenomas have an elevated number of somatostatin receptors.

Such tumors may be treated by the present invention by a somatostatin/lytic peptide combination.

#### ***Other Pituitary Adenomas***

Other ligands that may be used in a ligand/lytic peptide combination to treat other pituitary adenomas include TRH, MSH, GnRH, corticotropin-releasing hormone, growth hormone-releasing hormone, vasoactive intestinal polypeptide, and pituitary adenylate cyclase activating peptide. A short chain analog of  $\alpha$ MSH that may be used in place of MSH is Ser-Tyr-Cys-Met-Glu-His-Phe-Arg-Trp-Asn-Lys-Pro-Val-NH<sub>2</sub> (SEQ. ID NO. 10).

#### ***Other Endocrine-Related Diseases***

In other applications, the ligand/lytic peptide combination of the present invention may be used to treat endocrine-related diseases generally. Where a disease is causally related to dysfunction of cells having certain hormone receptors, cells with such receptors may be selectively inactivated by administering a combination of the hormone and a lytic peptide.

In an alternative approach, it has previously been noted that it is beneficial to reduce levels of LH and FSH in breast and prostate cancer patients. If the gonadotropes in the pituitary are selectively killed with a GnRH/lytic peptide combination, then the pituitary will no longer secrete LH and FSH. The reduced levels of these hormones thus resulting will help control the spread of the cancers. This alternative, indirect approach may be used in lieu of, or in addition to, treating the cancers directly with a LH/lytic peptide or FSH/lytic peptide

combination. Chronic administration of GnRH has previously been used to down-regulate its receptors, and thus effectively remove LH from circulation, resulting in "chemical castration" of prostatic cancer patients. However, GnRH and certain GnRH analogs also have direct effects on prostatic cell growth.

By analogy, it is well-established that surgical removal of the anterior pituitary is effective in treating sex hormone-related diseases. Chemical destruction of gonadotrophic cells in the pituitary through the present invention will therefore have similar effects on sex hormone-related diseases, but without the attendant risks and complications of surgery.

#### Examples 52-58

In these experiments we demonstrated *in vitro* lysis of human prostate cancer cell lines. LNCaP FGC and DU145 human prostate cancer cell lines were purchased from the American Type Culture Collection (ATCC, Rockville MD), ATCC accession numbers CRL 1740 and HTB-81, respectively. The LNCaP FGC adenocarcinoma cell line was originally obtained from a 50 year old male Caucasian. LNCaP FGC cells are sensitive to dihydrotestosterone and to estrogens (A+). The DU145 carcinoma was originally isolated from the brain of a 69 year old male Caucasian with metastatic carcinoma of the prostate; this cell line is not sensitive to steroid hormones (A-).

Cells were detached from culture flasks, and 1000 cells/well were transferred to 24 well culture plates. The cells were incubated for 24 hours with 10% calf serum. Cells were subsequently incubated without serum for 48 hours. Cells were then incubated for 22 hours with one of the following treatments:

**Treatment 52:** 10  $\mu$ M luteinizing hormone (LH)

**Treatment 53:** 30  $\mu$ M free hecate

**Treatment 54:** 90  $\mu$ M hecate-bLH

**Treatment 55:** 60  $\mu$ M hecate-bLH

**Treatment 56:** 50  $\mu$ M GnRH-hecate

**Treatment 57:** 10  $\mu$ M GnRH-hecate

**Treatment 58:** FSH pre-treatment, followed by 90  $\mu$ M hecate-bLH

Trypan blue exclusion was used to assess viability of the cells after treatment. The treatment that most consistently and effectively killed both the A+ and the A- cancer cell lines was the higher dose (50  $\mu$ M) of GnRH-hecate. The lower dose (10  $\mu$ M) of GnRH-hecate was equally effective against the androgen-insensitive DU145 cells. The DU145 cells were also killed by hecate alone. However, treatment with a lytic peptide alone may not be selective *in vivo* unless specific cell types are separately stimulated, for example by hormones controlling their



activity. The hecate-bLH conjugate killed almost all DU145 cells, but had little effect on A+ LNCaP. This result is consistent with specific binding of LH to DU145 cells but not to LNCaP cells. LH specifically binds DU145 cells, but we have not been able to consistently measure specific binding of LH to the A+ LNCaP cells. The LNCaP cells pre-treated with FSH were more sensitive to the hecate-bLH conjugate than those that were not pre-treated.

*Other Applications, including Treatment of Autoimmune Diseases, and Targeting of Abnormal Cells*

This invention may be used wherever it is desirable to specifically inhibit abnormal (or normal) cells that are driven by or are dependent on specific ligand interactions. As another example, this invention may be used in treating autoimmune diseases for which the antigen or epitope responsible for the autoimmune disease is known.

Specific immune responses are mediated by B-lymphocytes, T-lymphocytes, or both. When lymphocytes inappropriately attack "self" instead of "non-self," a variety of autoimmune diseases can result, some of which can have devastating consequences. Diseases that have been associated with autoimmunity include rheumatoid arthritis, juvenile rheumatoid arthritis, systemic lupus erythematosus, Addison's disease, Goodpasture's syndrome, autoimmune hemolytic anemia, Grave's disease, Hashimoto's thyroiditis, idiopathic thrombocytopenia purpura, insulin-dependent diabetes mellitus, myasthenia gravis, myocardial infarction, aplastic anemia, pernicious anemia, poststreptococcal glomerulonephritis, spontaneous infertility, ankylosing spondylitis, scleroderma, and Sjögrens' syndrome.

Whether mediated by T-cells or B-cells, autoimmune disease is characterized by lymphocytes with specific receptors for a self epitope that triggers their function -- i.e., antibody secretion, proliferation, secretion of cytotoxic factors, or secretion of inflammatory cytokines. These responses cause damage or destruction to self cells or organs.

The specific antigens and even epitopes that act as ligands to stimulate the lymphocytes have been identified for several autoimmune diseases, typically by the *in vitro* proliferative response they induce in lymphocytes. For example, thyrotropin has been implicated as the self-antigen recognized by lymphocytes in Hashimoto's Disease. Where the epitope is known, the autoimmune disease may be treated by administering a compound containing that epitope linked to a lytic peptide, which will selectively delete clones of the autoreactive lymphocytes.

There have previously been no general treatments for autoimmune diseases. Prior treatments have included cytotoxic compounds, and high doses of corticosteroids, both of which have risks in long-term therapy. Neither selectively targets autoreactive lymphocytes.

Certain abnormal cells (e.g., virally-infected cells such as HIV-infected cells, cancer cells) display surface receptors that are not found on normal cells. In some cases, these receptors are encoded by viral nucleic acids. Ligands for these receptors, such as monoclonal antibodies to those receptors, or the receptor/ligand pairs shown in Table 2 of D. Fitzgerald *et al.*, "Targeted Toxin Therapy for the Treatment of Cancer," *J. Natl. Cancer Inst.*, vol. 81, pp. 1455-1463, may be used in the ligand/lytic peptide combination of the present invention to selectively destroy cells displaying the receptor. Destruction of such a virally-infected cell, for example, before completion of the viral maturation cycle results in the release of incomplete, non-infectious viral particles, thereby treating the viral infection. Destruction of such a cancer cell prevents further metastasis. Where an antibody is used as the ligand, it will often be preferable to administer the antibody and the lytic peptide sequentially, rather than linked to one another. Complement and other responses to the bound antibodies make the cells more susceptible to attack by the lytic peptides.

#### *Lytic Peptides Useful in the Present Invention*

It is believed (without wishing to be bound by this theory) that lytic peptides act by disrupting cell membranes. "Resting" eukaryotic cells protect themselves through their ability to repair the resulting membrane damage. By contrast, activated cells (e.g., cells stimulated by GnRH) are unable (or less able) to repair damaged membranes. Because GnRH-activated pituitary cells have a diminished capacity to repair membranes, they are preferentially destroyed by lytic peptides, while adjacent non-activated cells repair their membranes and survive.

Although the embodiments of this invention that have been tested to date have used hecate as the effector lytic peptide, this invention will work with a combination of a ligand with other lytic peptides as well. Many lytic peptides are known in the art and include, for example, those mentioned in the references cited in the following discussion.

Lytic peptides are small, basic peptides. Native lytic peptides appear to be major components of the antimicrobial defense systems of a number of animal species, including those of insects, amphibians, and mammals. They typically comprise 23-39 amino acids, although they can be smaller. They have the potential for forming amphipathic alpha-helices. See Boman *et al.*, "Humoral immunity in *Cecropia* pupae," *Curr. Top. Microbiol. Immunol.* vol. 94/95, pp. 75-91 (1981); Boman *et al.*, "Cell-free immunity in insects," *Annu. Rev. Microbiol.*, vol. 41, pp. 103-126 (1987); Zasloff, "Magainins, a class of antimicrobial

peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial DNA sequence of a precursor," *Proc. Natl. Acad. Sci. USA*, vol. 84, pp. 3628-3632 (1987); Ganz *et al.*, "Defensins natural peptide antibiotics of human neutrophils," *J. Clin. Invest.*, vol. 76, pp. 1427-1435 (1985); and Lee *et al.*, "Antibacterial peptides from pig intestine: isolation of a mammalian cecropin," *Proc. Natl. Acad. Sci. USA*, vol. 86, pp. 9159-9162 (1989).

Known amino acid sequences for lytic peptides may be modified to create new peptides that would also be expected to have lytic activity by substitutions of amino acid residues that preserve the amphipathic nature of the peptides (e.g., replacing a polar residue with another polar residue, or a non-polar residue with another non-polar residue, etc.); by substitutions that preserve the charge distribution (e.g., replacing an acidic residue with another acidic residue, or a basic residue with another basic residue, etc.); or by lengthening or shortening the amino acid sequence while preserving its amphipathic character or its charge distribution. Lytic peptides and their sequences are disclosed in Yamada *et al.*, "Production of recombinant sarcotoxin IA in *Bombyx mori* cells," *Biochem. J.*, vol. 272, pp. 633-666 (1990); Taniai *et al.*, "Isolation and nucleotide sequence of cecropin B cDNA clones from the silkworm, *Bombyx mori*," *Biochimica Et Biophysica Acta*, vol. 1132, pp. 203-206 (1992); Boman *et al.*, "Antibacterial and antimalarial properties of peptides that are cecropin-melittin hybrids," *Febs Letters*, vol. 259, pp. 103-106 (1989); Tessier *et al.*, "Enhanced secretion from insect cells of a foreign protein fused to the honeybee melittin signal peptide," *Gene*, vol. 98, pp. 177-183 (1991); Blondelle *et al.*, "Hemolytic and antimicrobial activities of the twenty-four individual omission analogs of melittin," *Biochemistry*, vol. 30, pp. 4671-4678 (1991); Andreu *et al.*, "Shortened cecropin A-melittin hybrids. Significant size reduction retains potent antibiotic activity," *Febs Letters*, vol. 296, pp. 190-194 (1992); Macias *et al.*, "Bactericidal activity of magainin 2: use of lipopolysaccharide mutants," *Can. J. Microbiol.*, vol. 36, pp. 582-584 (1990); Rana *et al.*, "Interactions between magainin-2 and *Salmonella typhimurium* outer membranes: effect of Lipopolysaccharide structure," *Biochemistry*, vol. 30, pp. 5858-5866 (1991); Diamond *et al.*, "Airway epithelial cells are the site of expression of a mammalian antimicrobial peptide gene," *Proc. Natl. Acad. Sci. USA*, vol. 90, pp. 4596 ff (1993); Selsted *et al.*, "Purification, primary structures and antibacterial activities of  $\beta$ -defensins, a new family of antimicrobial peptides from bovine neutrophils," *J. Biol. Chem.*, vol. 268, pp. 6641 ff (1993); Tang *et al.*, "Characterization of the disulfide motif in BNBD-12, an antimicrobial  $\beta$ -defensin peptide from bovine neutrophils," *J. Biol. Chem.*, vol. 268, pp. 6649 ff (1993); Lehrer *et al.*, *Blood*, vol. 76, pp. 2169-2181 (1990); Ganz *et al.*, *Sem. Resp. Infect. I.*, pp. 107-117 (1986); Kagan *et al.*, *Proc. Natl. Acad. Sci. USA*, vol. 87, pp. 210-214 (1990); Wade *et al.*, *Proc. Natl. Acad. Sci. USA*, vol. 87, pp. 4761-4765 (1990);

Romeo *et al.*, *J. Biol. Chem.*, vol. 263, pp. 9573-9575 (1988); Jaynes *et al.*, "Therapeutic Antimicrobial Polypeptides, Their Use and Methods for Preparation," WO 89/00199 (1989); Jaynes, "Lytic Peptides, Use for Growth, Infection and Cancer," WO 90/12866 (1990); Berkowitz, "Prophylaxis and Treatment of Adverse Oral Conditions with Biologically Active Peptides," WO 93/01723 (1993).

Families of naturally-occurring lytic peptides include the cecropins, the defensins, the sarcotoxins, the melittins, and the magainins. Boman and coworkers in Sweden performed the original work on the humoral defense system of *Hyalophora cecropia*, the giant silk moth, to protect itself from bacterial infection. See Hultmark *et al.*, "Insect immunity. Purification of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*," *Eur. J. Biochem.*, vol. 106, pp. 7-16 (1980); and Hultmark *et al.*, "Insect immunity. Isolation and structure of cecropin D. and four minor antibacterial components from *cecropia* pupae," *Eur. J. Biochem.*, vol. 127, pp. 207-217 (1982).

Infection in *H. cecropia* induces the synthesis of specialized proteins capable of disrupting bacterial cell membranes, resulting in lysis and cell death. Among these specialized proteins are those known collectively as cecropins. The principal cecropins -- cecropin A, cecropin B, and cecropin D -- are small, highly homologous, basic peptides. In collaboration with Merrifield, Boman's group showed that the amino-terminal half of the various cecropins contains a sequence that will form an amphipathic alpha-helix. Andrequ *et al.*, "N-terminal analogues of cecropin A: synthesis, antibacterial activity, and conformational properties," *Biochem.*, vol. 24, pp. 1683-1688 (1985). The carboxy-terminal half of the peptide comprises a hydrophobic tail. See also Boman *et al.*, "Cell-free immunity in *Cecropia*," *Eur. J. Biochem.*, vol. 201, pp. 23-31 (1991).

A cecropin-like peptide has been isolated from porcine intestine. Lee *et al.*, "Antibacterial peptides from pig intestine: isolation of a mammalian cecropin," *Proc. Natl. Acad. Sci. USA*, vol. 86, pp. 9159-9162 (1989).

Cecropin peptides have been observed to kill a number of animal pathogens other than bacteria. See Jaynes *et al.*, "In Vitro Cytocidal Effect of Novel Lytic Peptides on *Plasmodium falciparum* and *Trypanosoma cruzi*," FASEB, 2878-2883 (1988); Arrowood *et al.*, "Hemolytic properties of lytic peptides active against the sporozoites of *Cryptosporidium parvum*," *J. Protozool.*, vol. 38, No. 6, pp. 161S-163S (1991); and Arrowood *et al.*, "In vitro activities of lytic peptides against the sporozoites of *Cryptosporidium parvum*," *Antimicrob. Agents Chemother.*, vol. 35, pp. 224-227 (1991). However, normal mammalian cells do not appear to be adversely affected by cecropins, even at high concentrations. See Jaynes *et al.*, "In vitro effect of lytic peptides on normal and transformed mammalian cell lines," *Peptide*

*Research*, vol. 2, No. 2, pp. 1-5 (1989); and Reed *et al.*, "Enhanced in vitro growth of murine fibroblast cells and preimplantation embryos cultured in medium supplemented with an amphipathic peptide," *Mol. Reprod. Devel.*, vol. 31, No. 2, pp. 106-113 (1992).

Defensins, originally found in mammals, are small peptides containing six to eight cysteine residues. Ganz *et al.*, "Defensins natural peptide antibiotics of human neutrophils," *J. Clin. Invest.*, vol. 76, pp. 1427-1435 (1985). Extracts from normal human neutrophils contain three defensin peptides: human neutrophil peptides HNP-1, HNP-2, and HNP-3. Defensin peptides have also been described in insects and higher plants. Dimarcq *et al.*, "Insect immunity: expression of the two major inducible antibacterial peptides, defensin and diptericin, in *Phormia terranvae*," *EMBO J.*, vol. 9, pp. 2507-2515 (1990); Fisher *et al.*, *Proc. Natl. Acad. Sci. USA*, vol. 84, pp. 3628-3632 (1987).

Slightly larger peptides called sarcotoxins have been purified from the fleshfly *Sarcophaga peregrina*. Okada *et al.*, "Primary structure of sarcotoxin I, an antibacterial protein induced in the hemolymph of *Sarcophaga peregrina* (flesh fly) larvae," *J. Biol. Chem.*, vol. 260, pp. 7174-7177 (1985). Although highly divergent from the cecropins and defensins, the sarcotoxins presumably have a similar antibiotic function.

Other lytic peptides have been found in amphibians. Gibson and collaborators isolated two peptides from the African clawed frog, *Xenopus laevis*, peptides which they named PGS and Gly<sup>10</sup>Lys<sup>22</sup>PGS. Gibson *et al.*, "Novel peptide fragments originating from PGL<sub>2</sub> and the caerulein and xenopsin precursors from *Xenopus laevis*," *J. Biol. Chem.*, vol. 261, pp. 5341-5349 (1986); and Givannini *et al.*, "Biosynthesis and degradation of peptides derived from *Xenopus laevis* prohormones," *Biochem. J.*, vol. 243, pp. 113-120 (1987). Zasloff showed that the *Xenopus*-derived peptides have antimicrobial activity, and renamed them magainins. Zasloff, "Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial DNA sequence of a precursor," *Proc. Natl. Acad. Sci. USA*, vol. 84, pp. 3628-3632 (1987).

Synthesis of nonhomologous analogs of different classes of lytic peptides has been reported to reveal that a positively charged, amphipathic sequence containing at least 20 amino acids appeared to be a requirement for lytic activity in some classes of peptides. Shiba *et al.*, "Structure-activity relationship of Lepidopteran, a self-defense peptide of *Bombyx more*," *Tetrahedron*, vol. 44, No. 3, pp. 787-803 (1988). Other work has shown that smaller peptides can also be lytic. See McLaughlin *et al.*, cited below.

Cecropins have been shown to target pathogens or compromised cells selectively, without affecting normal host cells. The synthetic lytic peptide known as S-1 (or Shiva 1) has been shown to destroy intracellular *Brucella abortus*-, *Trypanosoma cruzi*-, *Cryptosporidium*

*parvum*-, and infectious bovine herpes virus I (IBR)-infected host cells, with little or no toxic effects on noninfected mammalian cells. See Jaynes *et al.*, "In vitro effect of lytic peptides on normal and transformed mammalian cell lines," *Peptide Research*, vol. 2, No. 2, pp. 1-5 (1989); Wood *et al.*, "Toxicity of a Novel Antimicrobial Agent to Cattle and Hamster cells *In vitro*," Proc. Ann. Amer. Soc. Anim. Sci., Utah State University, Logan, UT. *J. Anim. Sci. (Suppl. 1)*, vol. 65, p. 380 (1987); Arrowood *et al.*, "Hemolytic properties of lytic peptides active against the sporozoites of *Cryptosporidium parvum*," *J. Protozool.*, vol. 38, No. 6, pp. 161S-163S (1991); Arrowood *et al.*, "In vitro activities of lytic peptides against the sporozoites of *Cryptosporidium parvum*," *Antimicrob. Agents Chemother.*, vol. 35, pp. 224-227 (1991); and Reed *et al.*, "Enhanced in vitro growth of murine fibroblast cells and preimplantation embryos cultured in medium supplemented with an amphipathic peptide," *Mol. Reprod. Devel.*, vol. 31, No. 2, pp. 106-113 (1992).

Morvan *et al.*, "In vitro activity of the antimicrobial peptide magainin 1 against *Bonamia ostreae*, the intrahemocytic parasite of the flat oyster *Ostrea edulis*," *Mol. Mar. Biol.*, vol. 3, pp. 327-333 (1994) reports the *in vitro* use of a magainin to selectively reduce the viability of the parasite *Bonamia ostreae* at doses that did not affect cells of the flat oyster *Ostrea edulis*.

Also of interest are the synthetic peptides disclosed in the following pending patent applications, peptides that have lytic activity with as few as 10-14 amino acid residues: McLaughlin *et al.*, "Amphipathic Peptides," United States patent application serial number 08/681,075, filed July 22, 1996; and Mark L. McLaughlin *et al.*, "Short Amphipathic Peptides with Activity against Bacteria and Intracellular Pathogens," United States patent application serial number 08/796,123, filed February 6, 1997.

Lytic peptides such as are known generally in the art may be used in practicing the present inventions. Selective toxicity to ligand-activated cells is desirable, especially when the ligand and peptide are administered separately. Selective toxicity is less important when the ligand and peptide are linked to one another, because in that case the peptide is effectively concentrated in the immediate vicinity of cells having receptors for the ligand.

Examples of such peptides are those designated D1A21 (SEQ. ID NO. 5), D2A21 (SEQ. ID NO. 6), D5C (SEQ. ID NO. 7), and D5C1 (SEQ. ID NO. 8). These peptides and other lytic peptides suitable for use in the present invention are disclosed in Jaynes, "Methods for the Design of Amphipathic Peptides Having Enhanced Biological Activities," United States provisional patent application serial number 60/027,628, filed October 4, 1996. In trials to date using these peptides alone (i.e., one of these four peptides without an associated ligand), *in vitro* LD<sub>50</sub> values against human prostate cancer cell lines have ranged from about 0.57  $\mu$ M

to about 1.61  $\mu$ M. In trials to date using D2A21 alone (i.e., without an associated ligand) LD<sub>50</sub> values against human breast, bladder, colon, cervix, lung, colon, and skin cancer cell lines have ranged from about 0.28  $\mu$ M to about 3.1  $\mu$ M. For comparison, LD<sub>50</sub> has been measured to be greater than 100  $\mu$ M for each of D2A21, D5C, and D5C1 for each of the following types of normal, non-cancerous human cells: endothelial cells, fibroblasts, enteric cells, and keratinocytes. For D2A21, LD<sub>50</sub> has been measured to be about 100  $\mu$ M for human peripheral blood monocytes, and to be greater than 100  $\mu$ M for human peripheral blood T-cells.

Other GnRH analogs may be conjugated with a lytic peptide in accordance with this invention. Among the analogs that may be used as part of such a conjugate is l-LHRH-III (or l-GnRH-III), SEQ. ID NO. 16. This peptide has been reported to suppress growth of several cancer cells. See I. Mezö *et al.*, "Synthesis of Gonadotropin-Releasing Hormone III Analogs. Structure-Antitumor Activity Relationships," *J. Med. Chem.* vol. 40, pp. 3353-3358 (1997). The same l-LHRH-III selectively causes the release of FSH. See W. Yu *et al.*, "A hypothalamic follicle-stimulating hormone-releasing decapeptide in the rat," *Proc. Natl. Acad. Sci USA*, vol. 94, pp. 9499-9503 (1997); and United States patent application S.N. 08/869,153, filed June 4, 1997. Lytic peptide conjugates of l-LHRH-III will be useful as contraceptives, and in the treatment of cancers such as prostate cancers. Agonists of l-LHRH-III, such as are disclosed in United States patent application S.N. 08/869,153, may be used as well.

#### *Miscellaneous*

As used in the Claims, an "effective amount" of a composition is an amount sufficient to selectively kill the targeted cells in a background population of non-targeted cells. Where appropriate in context, an "effective amount" of a composition is also an amount that is sufficient to induce long-term contraception or sterility in an animal. Where appropriate in context, an "effective amount" of GnRH or l-LHRH-III is an amount sufficient to temporarily restore fertility in an animal that has been made sterile by destruction of gonadotropic cells. As used in the Claims, the term "animal" is intended to include both human and non-human metazoans.

The complete disclosures of all references cited in this specification are her by incorporated by reference; as are the full disclosures of United States provisional application 60/041,009, filed March 27, 1997; United States provisional application 60/057,456, filed

September 3, 1997; and of United States non-provisional application 08/869,153, filed June 4, 1997. In the event of an otherwise irreconcilable conflict, however, the present specification shall control.



## SEQUENCE LISTING

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- (iii) NUMBER OF SEQUENCES: 18
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## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (ix) FEATURE:  
(A) NAME/KEY: Peptide  
(B) LOCATION: 1..10  
(D) OTHER INFORMATION: /note= "Xaa in position 1 denotes pyro-glutamic acid. This sequence is GnRH."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Xaa His Trp Ser Tyr Gly Leu Arg Pro Gly  
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..23
- (D) OTHER INFORMATION: /note= "This sequence is hecate."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Phe Ala Leu Ala Leu Lys Ala Leu Lys Lys Ala Leu Lys Lys Leu Lys  
1 5 10 15

Lys Ala Leu Lys Lys Ala Leu  
20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..33
- (D) OTHER INFORMATION: /note= "This sequence is a modified  
GnRH/hecate fusion peptide."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gln His Trp Ser Tyr Gly Leu Arg Pro Gly Phe Ala Leu Ala Leu Lys  
1 5 10 15

Ala Leu Lys Lys Ala Leu Lys Lys Leu Lys Lys Ala Leu Lys Lys Ala  
20 25 30

Leu

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION: 1..33

(D) OTHER INFORMATION: /note= "This sequence is a  
hecate/modified GnRH fusion peptide."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Phe Ala Leu Ala Leu Lys Ala Leu Lys Lys Ala Leu Lys Lys Leu Lys  
1 5 10 15

Lys Ala Leu Lys Lys Ala Leu Gln His Trp Ser Tyr Gly Leu Arg Pro  
20 25 30

Gly

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION: 1..23

(D) OTHER INFORMATION: /note= "This sequence is D1A21."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Phe Ala Phe Ala Phe Lys Ala Phe Lys Lys Ala Phe Lys Lys Phe Lys  
1 5 10 15

Lys Ala Phe Lys Lys Ala Phe  
20

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION: 1..23

(D) OTHER INFORMATION: /note= "This sequence is D2A21."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Phe Ala Lys Lys Phe Ala Lys Lys Phe Lys Lys Phe Ala Lys Lys Phe  
1 5 10 15

Ala Lys Phe Ala Phe Ala Phe  
20

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Peptide  
(B) LOCATION: 1..27  
(D) OTHER INFORMATION: /note= "This sequence is D5C."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys Arg Lys Arg Ala Val Lys Arg Val Gly Arg Arg Leu Lys Lys Leu  
1 5 10 15  
Ala Arg Lys Ile Ala Arg Leu Gly Val Ala Phe  
20 25

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Peptide  
(B) LOCATION: 1..37  
(D) OTHER INFORMATION: /note= "This sequence is D5C1."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Lys Arg Lys Arg Ala Val Lys Arg Val Gly Arg Arg Leu Lys Lys Leu  
1 5 10 15  
Ala Arg Lys Ile Ala Arg Leu Gly Val Ala Lys Leu Ala Gly Leu Arg  
20 25 30  
Ala Val Leu Lys Phe  
35

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Peptide  
(B) LOCATION: 1..10  
(D) OTHER INFORMATION: /note= "This sequence is a modified GnRH."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gln His Trp Ser Tyr Gly Leu Arg Pro Gly  
1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION: 1..13

(D) OTHER INFORMATION: /note= "This sequence is a modified  
alpha-MSH."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ser Tyr Cys Met Glu His Phe Arg Trp Asn Lys Pro Val  
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION: 1..15

(D) OTHER INFORMATION: /note= "This sequence is bLH."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ser Tyr Ala Val Ala Leu Ser Cys Gln Cys Ala Leu Cys Arg Arg  
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION: 1..38

(D) OTHER INFORMATION: /note= "This sequence is a  
hecate-bLH fusion peptide."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Phe Ala Leu Ala Leu Lys Ala Leu Lys Lys Ala Leu Lys Lys Leu Lys  
1 5 10 15

Lys Ala Leu Lys Lys Ala Leu Ser Tyr Ala Val Ala Leu Ser Cys Gln  
20 25 30

Cys Ala Leu Cys Arg Arg  
35

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..10
- (D) OTHER INFORMATION: /note= "Xaa in position 1 denotes  
pyro-glutamic acid. Xaa in position 6 denotes  
D-lysine. This sequence is D-Lys-6 GnRH."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Xaa His Trp Ser Tyr Xaa Leu Arg Pro Gly  
1 5 10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..10
- (D) OTHER INFORMATION: /note= "Xaa in position 1 denotes  
pyro-glutamic acid. Xaa in position 6 denotes  
acyl-D-lysine."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Xaa His Trp Ser Tyr Xaa Leu Arg Pro Gly  
1 5 10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Peptide  
(B) LOCATION: 1..33  
(D) OTHER INFORMATION: /note= "Xaa in position 1 denotes  
pyro-glutamic acid. This sequence is an  
1-LHRH-III/hecate fusion peptide."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Xaa His Trp Ser His Asp Trp Lys Pro Gly Phe Ala Leu Ala Leu Lys  
1 5 10 15  
Ala Leu Lys Lys Ala Leu Lys Lys Leu Lys Lys Ala Leu Lys Lys Ala  
20 25 30  
Leu

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Peptide  
(B) LOCATION: 1..10  
(D) OTHER INFORMATION: /note= "Xaa in position 1 denotes  
pyro-glutamic acid. This sequence is 1-LHRH-III."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Xaa His Trp Ser His Asp Trp Lys Pro Gly  
1 5 10

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Peptide  
(B) LOCATION: 1..10  
(D) OTHER INFORMATION: /note= "Xaa in position 1 denotes  
pyro-glutamic acid. This sequence is chicken I  
GnRH."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Xaa His Trp Ser Tyr Gly Leu Gln Pro Gly  
1 5 10

## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Peptide  
(B) LOCATION: 1..10  
(D) OTHER INFORMATION: /note= "Xaa in position 1 denotes  
pyro-glutamic acid. This sequence is chicken II  
GnRH."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Xaa His Trp Ser His Gly Trp Tyr Pro Gly  
1                      5                      10



What is claimed:

1           1.     A compound comprising: (a) a hormone domain selected from the group  
2 consisting of gonadotropin-releasing hormone, l-LHRH-III, bLH, estrogen, testosterone,  
3 luteinizing hormone, chorionic gonadotropin, follicle stimulating hormone, melanocyte-  
4 stimulating hormone, estradiol, dopamine, somatostatin, and analogues of these hormones; and  
5 (b) a lytic peptide domain.

1           2.     A compound as recited in Claim 1, wherein said hormone domain is bonded  
2 directly to said lytic peptide domain, without an intermediate linking domain joining said  
3 hormone domain to said lytic peptide domain.

1           3.     A compound as recited in Claim 1, wherein said lytic peptide domain is  
2 selected from the group consisting of a cecropin peptide, a melittin peptide, a defensin  
3 peptide, a magainin peptide, a sarcotoxin peptide, and analogs of said peptides.

1           4.     A compound as recited in Claim 1, wherein said lytic peptide domain  
2 comprises hecate.

1           5.     A compound as recited in Claim 1, wherein said hormone domain comprises l-  
2 LHRH-III.

1           6.     A compound as recited in Claim 1, wherein said hormone domain comprises  
2 gonadotropin-releasing hormone.

1           7.     A compound as recited in Claim 1, wherein said compound has the sequence  
2 SEQ. ID NO. 3 or SEQ. ID NO. 4.

1           8.     A compound as recited in Claim 1, wherein said compound has the sequence  
2 SEQ. ID NO. 12 or SEQ. ID NO. 15.

1           9.     A compound as recited in Claim 1, wherein said hormone domain comprises  
2 estrogen.

1           **10.**    A compound as recited in Claim 1, wherein said hormone domain comprises  
2    testosterone.

1           **11.**    A compound as recited in Claim 1, wherein said hormone domain comprises  
2    luteinizing hormone.

1           **12.**    A compound as recited in Claim 1, wherein said hormone domain comprises  
2    chorionic gonadotropin.

1           **13.**    A compound as recited in Claim 1, wherein said hormone domain comprises  
2    follicle stimulating hormone.

1           **14.**    A compound as recited in Claim 1, wherein said hormone domain comprises  
2    melanocyte-stimulating hormone.

1           **15.**    A compound as recited in Claim 1, wherein said hormone domain comprises  
2    estradiol.

1           **16.**    A compound as recited in Claim 1, wherein said hormone domain comprises  
2    dopamine.

1           **17.**    A compound as recited in Claim 1, wherein said hormone domain comprises  
2    somatostatin.

1           **18.**    A compound as recited in Claim 1, wherein said hormone domain, or said  
2    lytic peptide domain, or both comprise D-conformation amino acid residues.

1           **19.**    A compound as recited in Claim 18, additionally comprising a carrier domain  
2    to facilitate uptake by the intestine when the compound is administered orally.

1           **20.**    A compound as recited in Claim 19, wherein said carrier domain comprises a  
2    vitamin B<sub>12</sub> domain.

1           **21.**    A method for producing long-term contraception or sterility in an animal,  
2           comprising administering to the animal an effective amount of: (a) a hormone selected from  
3           the group consisting of gonadotropin-releasing hormone, bLH, and l-LHRH-III, and (b) an  
4           effective amount of a lytic peptide.

1           **22.**    A method as recited in Claim 21, wherein the lytic peptide is administered  
2           after the hormone is administered.

1           **23.**    A method as recited in Claim 21, wherein the animal is a mammal.

1           **24.**    A method as recited in Claim 21, wherein the animal is a bird.

1           **25.**    A method as recited in Claim 24, wherein the bird is a chicken or a turkey.

1           **26.**    A method as recited in Claim 21, wherein the animal is an insect.

1           **27.**    A method as recited in Claim 26, wherein the hormone and the lytic peptide  
2           are expressed by an exogenous gene in a plant consumed by the insect.

1           **28.**    A method as recited in Claim 21, wherein the hormone, or the lytic peptide,  
2           or both comprise D-conformation amino acid residues.

1           **29.**    A method as recited in Claim 28, wherein the compound containing  
2           D-conformation amino acid residues additionally comprising a carrier domain to facilitate  
3           uptake by the intestine when the compound is administered orally.

1           **30.**    A method as recited in Claim 29, wherein the carrier domain comprises a  
2           vitamin B<sub>12</sub> domain.

1           **31.**    A method for producing long-term contraception or sterility in an animal,  
2           comprising administering to the animal an effective amount of a compound comprising a  
3           hormone domain and a lytic peptide domain, wherein said hormone domain is selected from  
4           the group consisting of gonadotropin-releasing hormone, l-LHRH-III, and bLH.

1           **32.**    A method as recited in Claim 31, wherein the hormone domain is bonded  
2 directly to the lytic peptide domain, without an intermediate linking domain joining the  
3 hormone domain to the lytic peptide domain.

1           **33.**    A method as recited in Claim 31, wherein the lytic peptide domain is selected  
2 from the group consisting of a cecropin peptide, a melittin peptide, a defensin peptide, a  
3 magainin peptide, a sarcotoxin peptide, and analogs of said peptides.

1           **34.**    A method as recited in Claim 31, wherein the lytic peptide domain comprises  
2 hecate.

1           **35.**    A method as recited in Claim 31, wherein the compound has the sequence  
2 SEQ. ID NO. 3.

1           **36.**    A method as recited in Claim 31, wherein the compound has the sequence  
2 SEQ. ID NO. 4.

1           **37.**    A method as recited in Claim 31, wherein the compound has the sequence  
2 SEQ. ID NO. 12 or SEQ. ID NO. 15.

1           **38.**    A method as recited in Claim 31, wherein the animal is a mammal.

1           **39.**    A method as recited in Claim 31, wherein the animal is a bird.

1           **40.**    A method as recited in Claim 39, wherein the bird is a chicken or a turkey.

1           **41.**    A method as recited in Claim 31, wherein the animal is an insect.

1           **42.**    A method as recited in Claim 41, wherein the peptide is expressed by an  
2 exogenous gene in a plant consumed by the insect.

1           **43.**    A method of temporarily restoring fertility in a mammal that had been made  
2 sterile by the selective destruction of gonadotropes in the pituitary, comprising administering  
3 to the mammal an effective amount of gonadotropin-releasing hormone or LHRH-III.

1           **44.**    A method as recited in Claim 43, wherein fertility is restored in a mammal  
2           that had previously been made sterile by administering to the animal an effective amount of:  
3           (a) a hormone selected from the group consisting of gonadotropin-releasing hormone, l-  
4           LHRH-III, and bLH, and (b) an effective amount of a lytic peptide.

1           **45.**    A method as recited in Claim 43, wherein fertility is restored in a mammal  
2           that had previously been made sterile by administering to the animal an effective amount of a  
3           compound comprising a hormone domain and a lytic peptide domain, wherein said hormone  
4           domain is selected from the group consisting of gonadotropin-releasing hormone, l-LHRH-III,  
5           and bLH.

1           **46.**    A plant containing an exogenous gene that encodes a peptide comprising a  
2           hormone domain and a lytic peptide domain, wherein said hormone domain is selected from  
3           the group consisting of gonadotropin-releasing hormone, l-LHRH-III, and bLH.

1           **47.**    A plant containing a first exogenous gene that encodes gonadotropin-releasing  
2           hormone or that encodes l-LHRH or that encodes bLH, and a second exogenous gene that  
3           encodes a lytic peptide.

1           **48.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2           or ligand-dependent tumor in a mammal, comprising administering to the mammal an effective  
3           amount of the hormone or ligand on which the growth of the tumor depends, and an effective  
4           amount of a lytic peptide.

1           **49.**    A method as recited in Claim 48, wherein the lytic peptide is administered  
2           after the hormone or ligand is administered.

1           **50.**    A method as recited in Claim 48, wherein the hormone or ligand and the lytic  
2           peptide are each administered by administering to the mammal a compound in which the  
3           hormone or ligand and the lytic peptide are chemically bonded to one another.

1           **51.**    A method as recited in Claim 48, wherein the cell is part of an ovarian cancer,  
2           and wherein the hormone or ligand comprises estradiol.

1           **52.**    A method as recited in Claim 48, wherein the cell is part of a breast cancer,  
2           and wherein the hormone or ligand comprises estradiol.

1           **53.**    A method as recited in Claim 48, wherein the cell is part of a prostate cancer,  
2           and wherein the hormone or ligand comprises testosterone.

1           **54.**    A method as recited in Claim 48, wherein the cell is part of a prolactinoma,  
2           and wherein the hormone or ligand comprises dopamine.

1           **55.**    A method as recited in Claim 48, wherein the cell is part of a growth  
2           hormone-secreting adenoma, and wherein the hormone or ligand comprises growth hormone.

1           **56.**    A method as recited in Claim 48, wherein the cell is part of a thyrotropin-  
2           releasing hormone-secreting adenoma, and wherein the hormone or ligand comprises  
3           thyrotropin-releasing hormone.

1           **57.**    A method as recited in Claim 48, wherein the cell is part of a gonadotropin-  
2           secreting adenoma, and wherein the hormone or ligand comprises gonadotropin.

1           **58.**    A method as recited in Claim 48, wherein the cell is part of a growth  
2           hormone-secreting adenoma, and wherein the hormone or ligand comprises somatostatin.

1           **59.**    A method as recited in Claim 48, wherein the cell is part of a pituitary  
2           adenoma, and wherein the hormone or ligand is selected from the group consisting of  
3           gonadotropin-releasing hormone, l-LHRH-III, corticosteroid-releasing hormone, growth  
4           hormone-releasing hormone, vasoactive intestinal polypeptide, and pituitary adenylate cyclase  
5           activating peptide.

1           **60.**    A method as recited in Claim 48, wherein the cell is part of a breast cancer,  
2           and wherein the hormone or ligand comprises gonadotropin-releasing hormone or l-LHRH-III.

1           **61.**    A method as recited in Claim 48, wherein the cell is part of an ovarian cancer,  
2           and wherein the hormone or ligand comprises gonadotropin-releasing hormone, l-LHRH-III,  
3           or bLH.

1           **62.**    A method as recited in Claim 48, wherein the cell is part of a prostate cancer,  
2           and wherein the hormone or ligand comprises gonadotropin-releasing hormone or l-LHRH-III.

1           **63.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2           tumor in a mammal, comprising administering to the mammal an effective amount of a  
3           compound as recited in Claim 1, wherein the hormone domain of the compound comprises the  
4           hormone on which the tumor is dependent.

1           **64.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2           tumor in a mammal, comprising administering to the mammal an effective amount of a  
3           compound as recited in Claim 2, wherein the hormone domain of the compound comprises the  
4           hormone on which the tumor is dependent.

1           **65.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2           tumor in a mammal, comprising administering to the mammal an effective amount of a  
3           compound as recited in Claim 3, wherein the hormone domain of the compound comprises the  
4           hormone on which the tumor is dependent.

1           **66.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2           tumor in a mammal, comprising administering to the mammal an effective amount of a  
3           compound as recited in Claim 4, wherein the hormone domain of the compound comprises the  
4           hormone on which the tumor is dependent.

1           **67.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2           tumor in a mammal, comprising administering to the mammal an effective amount of a  
3           compound as recited in Claim 5, wherein the hormone domain of the compound comprises the  
4           hormone on which the tumor is dependent.

1           **68.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2           tumor in a mammal, comprising administering to the mammal an effective amount of a  
3           compound as recited in Claim 6, wherein the hormone domain of the compound comprises the  
4           hormone on which the tumor is dependent.

1           **69.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2    tumor in a mammal, comprising administering to the mammal an effective amount of a  
3    compound as recited in Claim 7, wherein the hormone domain of the compound comprises the  
4    hormone on which the tumor is dependent.

1           **70.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2    tumor in a mammal, comprising administering to the mammal an effective amount of a  
3    compound as recited in Claim 8, wherein the hormone domain of the compound comprises the  
4    hormone on which the tumor is dependent.

1           **71.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2    tumor in a mammal, comprising administering to the mammal an effective amount of a  
3    compound as recited in Claim 9, wherein the hormone domain of the compound comprises the  
4    hormone on which the tumor is dependent.

1           **72.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2    tumor in a mammal, comprising administering to the mammal an effective amount of a  
3    compound as recited in Claim 10, wherein the hormone domain of the compound comprises  
4    the hormone on which the tumor is dependent.

1           **73.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2    tumor in a mammal, comprising administering to the mammal an effective amount of a  
3    compound as recited in Claim 11, wherein the hormone domain of the compound comprises  
4    the hormone on which the tumor is dependent.

1           **74.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2    tumor in a mammal, comprising administering to the mammal an effective amount of a  
3    compound as recited in Claim 12, wherein the hormone domain of the compound comprises  
4    the hormone on which the tumor is dependent.

1           **75.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2    tumor in a mammal, comprising administering to the mammal an effective amount of a  
3    compound as recited in Claim 13, wherein the hormone domain of the compound comprises  
4    the hormone on which the tumor is dependent.



1           **76.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2 tumor in a mammal, comprising administering to the mammal an effective amount of a  
3 compound as recited in Claim 14, wherein the hormone domain of the compound comprises  
4 the hormone on which the tumor is dependent.

1           **77.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2 tumor in a mammal, comprising administering to the mammal an effective amount of a  
3 compound as recited in Claim 15, wherein the hormone domain of the compound comprises  
4 the hormone on which the tumor is dependent.

1           **78.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2 tumor in a mammal, comprising administering to the mammal an effective amount of a  
3 compound as recited in Claim 16, wherein the hormone domain of the compound comprises  
4 the hormone on which the tumor is dependent.

1           **79.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2 tumor in a mammal, comprising administering to the mammal an effective amount of a  
3 compound as recited in Claim 17, wherein the hormone domain of the compound comprises  
4 the hormone on which the tumor is dependent.

1           **80.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2 tumor in a mammal, comprising administering to the mammal an effective amount of a  
3 compound as recited in Claim 18, wherein the hormone domain of the compound comprises  
4 the hormone on which the tumor is dependent.

1           **81.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2 tumor in a mammal, comprising administering to the mammal an effective amount of a  
3 compound as recited in Claim 19, wherein the hormone domain of the compound comprises  
4 the hormone on which the tumor is dependent.

1           **82.**    A method for killing or inhibiting the growth of a cell in a hormone-dependent  
2 tumor in a mammal, comprising administering to the mammal an effective amount of a  
3 compound as recited in Claim 20, wherein the hormone domain of the compound comprises  
4 the hormone on which the tumor is dependent.

1           **83.**    A method for killing or inhibiting the growth of a cell in a mammal, wherein  
2           the activity of the cell is dependent on the binding of a receptor on the cell surface to a ligand,  
3           said method comprising administering to the mammal an effective amount of the ligand on  
4           which the activity of the cell depends, and an effective amount of a lytic peptide.

1           **84.**    A method as recited in Claim 83, wherein the lytic peptide is administered  
2           after the ligand is administered.

1           **85.**    A method as recited in Claim 84, wherein the ligand and the lytic peptide are  
2           each administered by administering to the mammal a compound in which the ligand and the  
3           lytic peptide are chemically bonded to one another.

1           **86.**    A method as recited in Claim 83, wherein the cell is a lymphocyte responsible  
2           for an autoimmune reaction, and wherein the ligand comprises an epitope to which the  
3           lymphocyte selectively binds.

1           **87.**    A method as recited in Claim 83, wherein the cell is a virally-infected cell that  
2           displays a surface receptor not displayed by otherwise similar, but uninfected cells, and  
3           wherein the ligand selectively binds to the surface receptor.

1           **88.**    A method for inhibiting the reproductive ability of an insect, comprising  
2           administering to the insect an effective amount of a lytic peptide.

1           **89.**    A method as recited in Claim 88, wherein the lytic peptide is selected from the  
2           group consisting of a cecropin peptide, a melittin peptide, a defensin peptide, a magainin  
3           peptide, a sarcotoxin peptide, and analogs of said peptides.

1           **90.**    A method as recited in Claim 88, wherein the lytic peptide comprises L-  
2           hecate.

1           **91.**    A method as recited in Claim 88, wherein the lytic peptide comprises D-  
2           hecate.

1           **92.**    A method as recited in Claim 88, wherein the lytic peptide is expressed by an  
2           exogenous gene in a plant consumed by the insect.

- 1           **93.**    A method as recited in Claim 92, wherein the lytic peptide expressed by the  
2    plant comprises L-hecate.
- 1           **94.**    A plant containing an exogenous gene that encodes L-hecate.
- 1           **95.**    A method as recited in Claim 23, wherein the mammal is a dog.
- 1           **96.**    A method as recited in Claim 23, wherein the mammal is a cat.
- 1           **97.**    A method as recited in Claim 23, wherein the mammal is a cow or bull.
- 1           **98.**    A method as recited in Claim 23, wherein the mammal is a pig.
- 1           **99.**    A method as recited in Claim 23, wherein the mammal is a horse.
- 1           **100.**   A method as recited in Claim 23, wherein the mammal is a sheep.
- 1           **101.**   A method as recited in Claim 23, wherein the mammal is a human.
- 1           **102.**   A method as recited in Claim 21, wherein the animal is a mollusc.
- 1           **103.**   A method as recited in Claim 102, wherein the mollusc is a zebra mussel.
- 1           **104.**   A method as recited in Claim 102, wherein the mollusc is an oyster.
- 1           **105.**   A method as recited in Claim 38, wherein the mammal is a dog.
- 1           **106.**   A method as recited in Claim 38, wherein the mammal is a cat.
- 1           **107.**   A method as recited in Claim 38, wherein the mammal is a cow or bull.
- 1           **108.**   A method as recited in Claim 38, wherein the mammal is a pig.
- 1           **109.**   A method as recited in Claim 38, wherein the mammal is a horse.

- 1           **110.** A method as recited in Claim 38, wherein the mammal is a sheep.
- 1           **111.** A method as recited in Claim 38, wherein the mammal is a human.
- 1           **112.** A method as recited in Claim 31, wherein the animal is a mollusc.
- 1           **113.** A method as recited in Claim 112, wherein the mollusc is a zebra mussel.
- 1           **114.** A method as recited in Claim 112, wherein the mollusc is an oyster.
- 1           **115.** A method for selectively killing gonadotrophic cells in the pituitary of an  
2 animal, comprising administering to the animal: (a) an effective amount of a hormone  
3 selected from the group consisting of gonadotropin-releasing hormone and l-LHRH-III, and  
4 (b) an effective amount of a lytic peptide.
- 1           **116.** A method for selectively killing gonadotrophic cells in the pituitary of an  
2 animal, comprising administering to the animal an effective amount of a compound comprising  
3 a hormone domain and a lytic peptide domain, wherein said hormone domain is selected from  
4 the group consisting of gonadotropin-releasing hormone and l-LHRH-III.
- 1           **117.** A method for selectively killing neurons having gonadotrophic receptors in an  
2 animal, comprising administering to the animal: (a) an effective amount of a hormone  
3 selected from the group consisting of gonadotropin-releasing hormone, l-LHRH-III, and bLH,  
4 and (b) an effective amount of a lytic peptide.
- 1           **118.** A method for selectively killing neurons having gonadotrophic receptors in an  
2 animal, comprising administering to the animal an effective amount of a compound comprising  
3 a hormone domain and a lytic peptide domain, wherein said hormone domain is selected from  
4 the group consisting of gonadotropin-releasing hormone, l-LHRH-III, and bLH.
- 1           **119.** A method as recited in Claim 21, wherein the animal is sexually immature.
- 1           **120.** A method as recited in Claim 31, wherein the animal is sexually immature.
- 1           **121.** A method as recited in Claim 23, wherein the mammal is sexually immature.

1           **122.**   A method as recited in Claim 38, wherein the mammal is sexually immature.

1           **123.**   A method as recited in Claim 48, wherein the cell is part of an ovarian cancer,  
2   and wherein the hormone or ligand comprises l-LHRH-III.

1           **124.**   A method as recited in Claim 48, wherein the cell is part of a prostatic cancer,  
2   and wherein the hormone or ligand comprises l-LHRH-III.

1           **125.**   A method as recited in Claim 48, wherein the cell is part of a breast cancer,  
2   and wherein the hormone or ligand comprises l-LHRH-III.

1           **126.**   A method as recited in Claim 48, wherein the cell is part of an endometrial  
2   cancer, and wherein the hormone or ligand comprises l-LHRH-III.

1           **127.**   A compound as recited in Claim 1, wherein said hormone domain comprises  
2   bLH.

1           **128.**   A method as recited in Claim 48, wherein the cell is part of a testicular  
2   cancer, and wherein the hormone or ligand comprises gonadotropin-releasing hormone, l-  
3   LHRH-III, or bLH.

## INTERNATIONAL SEARCH REPORT

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## A. CLASSIFICATION OF SUBJECT MATTER

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US CL :530/324

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/324, 325, 326

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS Online

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,589,457 A (WILTBANK et al) 31 December 1996.	1-128
A,P	US 5,631,229 A (NETT et al) 20 May 1997, see entire document.	1-128

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Further documents are listed in the continuation of Box C.

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See patent family annex.

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<b>(54) Title:</b> ANALOGS OF GLYCOPROTEIN HORMONES HAVING ALTERED RECEPTOR BINDING SPECIFICITY AND ACTIVITY AND METHODS FOR PREPARING AND USING SAME  <b>(57) Abstract</b> <p>The present invention pertains to an <i>alpha</i>, <i>beta</i>-heterodimeric polypeptide having binding affinity to vertebrate luteinizing hormone (LH) receptors and vertebrate follicle stimulating hormone (FSH) receptors comprising a glycoprotein hormone <i>alpha</i>-subunit polypeptide and a non-naturally occurring <i>beta</i>-subunit polypeptide, wherein the <i>beta</i>-subunit polypeptide is a chain of amino acids comprising the following four joined subsequences: (a) a first subsequence homologous to the amino acid sequence of residues 1-93 of the <i>beta</i>-subunit selected from the group consisting of human chorionic gonadotrophin (hCG), vertebrate luteinizing hormone (LH), vertebrate follicle stimulating hormone (FSH), and vertebrate thyroid stimulating hormone (TSH); (b) a second subsequence homologous to the amino acid sequence of residues 94-97 of the <i>beta</i>-subunit selected from the group consisting of human chorionic gonadotrophin (hCG) and vertebrate luteinizing hormone (LH); (c) a third subsequence homologous to the amino acid sequence of residues 98-100 of the <i>beta</i>-subunit selected from the group consisting of human chorionic gonadotrophin (hCG), vertebrate luteinizing hormone (LH), vertebrate follicle stimulating hormone (FSH), and vertebrate thyroid stimulating hormone (TSH); and (d) a fourth subsequence homologous to the amino acid sequence of residues 101-110 of the <i>beta</i>-subunit of vertebrate follicle stimulating hormone.</p>		

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**ANALOGS OF GLYCOPROTEIN HORMONES  
HAVING ALTERED RECEPTOR BINDING SPECIFICITY AND ACTIVITY  
AND METHODS FOR PREPARING AND USING SAME**

15

**Field of the Invention**

The present invention relates to glycoprotein hormone analogs having altered receptor binding specificity and activity. Specifically, the invention pertains to alpha, beta-heterodimeric polypeptides having binding affinity to follicle stimulating hormone (FSH) receptors and luteinizing hormone (LH) receptors. The residues important for receptor binding and specificity are located between Cys93 and Cys100 and Cys100 and Cys110 of hCG. These residues correspond to hFSH residues 87-94 and 94-104. A wide variety of alpha, beta-heterodimeric polypeptides can be made to alter the LH and FSH receptor binding activity and specificity of the polypeptides without disrupting their abilities to form alpha, beta-heterodimers or react with antibodies. This invention also pertains to methods for preparing these alpha, beta-heterodimeric polypeptides and the pharmaceutical compositions in which they may be employed.

**The R productive Glycoprotein Hormones  
and Their Biological Actions.**

The glycoprotein hormone family consists of  
5 three alpha, beta heterodimeric glycoproteins found in  
the anterior pituitary gland where they are made and  
includes luteinizing hormone (LH), follicle stimulating  
hormone (FSH), and thyroid stimulating hormone (TSH). In  
10 some species, a glycoprotein hormone structurally similar  
to LH is found in the placenta wherein it is synthesized.  
In humans, this glycoprotein hormone is called human  
chorionic gonadotropin (hCG). In primates, significant  
quantities of all the hormones are also found as  
15 excretion products in urine. Urine from pregnant women  
serves as a convenient source of hCG. After menopause,  
when the secretion of LH and FSH from the anterior  
pituitary is greatly increased, significant quantities of  
LH and FSH are found in the urine and are termed human  
20 menopausal gonadotropins (hMG). Urine from menopausal  
women serves as an important source of LH and FSH  
activities. Both urinary hCG and hMG have important  
commercial uses. Unlike hCG, which interacts with LH  
receptors and only weakly with FSH receptors, hMG  
25 interacts with both LH and FSH receptors. The activity  
of hMG is due to the presence of multiple hormone species  
in the urinary extract.

Gonadotropins such as CG, LH, and FSH play a  
major role in the reproductive process while the  
30 structurally related hormone, TSH, is important for  
thyroid function. Both LH and FSH are essential for  
normal reproductive function and treatments to reduce LH  
activity result in infertility, termination of pregnancy,  
or both. FSH plays a crucial role in fertility. In the  
35 absence of sufficient FSH, women fail to have a normal  
menstrual cycle, i.e., a cycle in which a follicle  
develops to the point of ovulation. In polycystic  
ovarian disease, fertility can be restored by  
administration of FSH to women. The hormone hCG is

important for maintenance of pregnancy. In males, the absence of LH and FSH is associated with infertility. The hormone LH is required for puberty and, in its absence, there is a failure to acquire the sexual attributes and fertility of an adult. The clinical activities of these hormones are reviewed extensively in several standard textbooks including that by Yen and Jaffe (1).

Both hCG and LH bind to luteinizing hormone receptors (LHR). In the testis, LHR are found primarily in the Leydig cells. In the ovary, LHR are found primarily in thecal, FSH-stimulated granulosa, and luteal cells. The major role of LH is to stimulate the formation of steroid hormones including the androgens testosterone and androstenedione (Leydig and thecal cells) and progesterone (FSH-stimulated granulosa, thecal, and luteal cells). LH also causes ovulation of mature follicles. While hCG is normally produced only by the placenta during pregnancy, due to its high affinity for LH receptors, the ease with which it can be purified from urine, and its long biological half-life, hCG has been widely used as a substitute for LH. Important clinical uses for hCG include stimulation of fertility in males and induction of ovulation in females.

FSH binds to FSH receptors (FSHR) which are located primarily in the Sertoli cells of the testis and the granulosa cells of the ovaries. The primary roles of FSH are to stimulate the conversion of androgens (i.e., the steroids produced in response to LH stimulation of testicular Leydig cells and ovarian thecal cells) to estrogens, to promote the synthesis of inhibin and activin, to promote the development of Sertoli and granulosa cells, and to stimulate gamete maturation. The effect of FSH on granulosa cells leads to follicular maturation, a process during which the oocyte is prepared for ovulation and in which the granulosa cells acquire the ability to respond to LH. Follicle maturation is

essential for the ability of LH to induce ovulation. Initiation of spermatogenesis requires FSH in addition to LH.

5                   The differences in the effects of FSH and LH and the complex endocrine interactions between the two hormones cause them to have synergistic effects. For example, normal estrogen production is due to the effect of LH on androgen formation and the influence of FSH on  
10                   the conversion of androgens to estradiol. This process is regulated by negative and positive feedback mechanisms wherein estradiol can inhibit FSH secretion and increase LH secretion from the pituitary gland. For this reason, the ratio of LH/FSH activity as well as the absolute  
15                   hormone levels in blood are important for reproductive functions such as sperm production and ovulation of the proper number of oocytes during the menstrual and estrus cycles.

20                   **Uses of Glycoprotein Hormones  
and Desirability of Novel Hormone Analogs.**

                  Mixtures of FSH and LH activities (hMG) are routinely used in the treatment of human infertility, a  
25                   condition affecting approximately 10% of all couples (2, 3). This particular combination therapy is necessary because gonadal support of gamete maturation is dependent upon the synergistic actions of both FSH and LH. Current treatment protocols requiring FSH and LH activity utilize  
30                   urinary extracts from postmenopausal women. The use of these extracts is compromised by several factors including batch (4, 5) and supplier (6) variability, expense of treatment (7), and the risk of gonadal hyperstimulation, a potentially fatal side effect (8).  
35                   These limitations potentially would be overcome through the use of hormone analogs which combine FSH and LH activity in the same molecule. Although there is a naturally occurring hormone, equine lutropin (eLH) which exhibits both FSH and LH activity in certain nonequine

species (9), its practical application has been limited by side effects (8) and cross-species intolerance (3). Because the structural basis of the LH and FSH activity of eLH has not been understood, it has not been useful as a model for the engineering of mixed functions into hormones from other species. Further, it is not possible to reproducibly prepare eLH with different ratios of LH to FSH activity. In women with some forms of polycystic ovarian disease in which endogenous LH levels are elevated, it would be desirable to devise methods for producing glycoprotein hormone analogs which have a relatively higher ratio of FSH:LH activity. While protein engineering techniques have recently been reported to prepare analogs of hCG which bind with high affinity to FSH receptors (10), there has been no procedure for preparing glycoprotein hormones which bind well to LH and FSH receptors or which bind to LH and FSH receptors with a predictable ratio of FSH:LH activities.

Pathological changes in the ratios of FSH:LH are often associated with infertility (e.g., polycystic ovarian disease). Induction of ovulation can be influenced by the ratio of FSH:LH in serum (9) and it would be desirable to prepare analogs of glycoprotein hormones with any given ratio of LH/FSH activity. Presently, the only means of adjusting the ratios of LH to FSH in hormone preparations is to add FSH, LH, or hCG to them or to change the purification scheme. LH, FSH, and hCG have greatly differing half-lives and the ratio of hormone activity following in vivo administration of these preparations changes with time. Thus, mixtures of FSH and LH would gradually assume a higher ratio of FSH:LH activity following administration due to the longer half-life of FSH relative to LH. Since hCG has a much longer half-life than FSH, the ratio of FSH:LH activity of hFSH/hCG mixtures would gradually decrease after administration. It would be desirable to have analogs which contained a predefined ratio of LH to FSH activity in the same molecule. The ratio of FSH:LH

activity in analogs with the capacity to bind to both LH and FSH receptors is expected to remain relatively constant after hormone administration. Analogs containing sequences derived from LH and FSH would have relatively short half-lives. In contrast, since the half-life of hCG is much longer than that of LH or FSH, if one were to use hCG as the primary structural component of these analogs, it should be possible to make analogs with a very long half-life. This would reduce the amounts of the analog needed for a biological effect. Small amounts of these analogs with approximately equal LH and FSH activity would be expected to be useful for inducing ovulation in women with hypothalamic amenorrhea and in males who fail to undergo puberty. Small amounts of analogs with lower ratios of LH/FSH activity would be expected to be useful clinically in cases where some endogenous LH is present such as inducing ovulation in women with polycystic ovarian disease or for increasing spermatogenesis in azospermic males who have some circulating LH. Previously, we have shown that it is possible to produce hCG analogs having very low LH activity and very high FSH activity (10). While these are primarily useful as FSH analogs, their LH activity could be increased only if they were mixed with hCG. Analogs of the human hormones with significant intrinsic LH and FSH activities have not yet been devised. In addition, based on the existing knowledge of the structure and functions of the glycoprotein hormones reviewed below, there is no obvious strategy which can enable these analogs to be devised.

### Structures of the Glycoprotein Hormones

The structures of the glycoprotein hormones have been studied for many years and considerable information exists as to the relative roles of the hormone subunits (11). These hormones share a common alpha-subunit but differ in their hormone-specific beta-subunits which determine the biological and immunological

properties of each hormone. The sequences of the subunits were determined several years ago and were confirmed from the base sequences of the subunit cDNA which had been cloned from pituitary and placental libraries (12, 13). Substitution of the alpha-subunits of any one hormone for that of another does not change the receptor binding properties of the new hormone. Substitution of the beta-subunit is accompanied by a change in the receptor binding specificity of the resulting hormone. Thus, when FSH beta-subunit is substituted for the LH beta-subunit, the recombined hormone acquires the properties of FSH and loses those characteristics of LH.

Several attempts have been made to identify portions of the alpha- and beta-subunits of the hormones which are responsible for their unique biological properties. Earlier studies were based on chemical modifications of the hormones (11). Modifications were described which reduced the biological activities of the hormones but no analogs were prepared which had switched LH and FSH receptor binding specificities. Due to the complexity of the hormones, this approach was unable to identify amino acid residues which were involved in ligand binding specificity. In an attempt to simplify the problem of identifying residues which are involved in ligand binding specificity, some investigators prepared synthetic peptides corresponding to partial sequences of the alpha- or beta-subunits and monitored their abilities to inhibit binding of  $^{125}\text{I}$ -hCG and  $^{125}\text{I}$ -hFSH to LH and FSH receptors. Synthetic peptides corresponding to amino acid residues of hCG-beta 38-57 or hFSH-beta 31-52 appear to have higher abilities than most other peptides to bind to these receptors (14, 15, 16). However, they have extremely low affinities for the receptors, an observation which precludes their practical use.

A breakthrough in the ability to make and characterize glycoprotein hormone analogs came in 1985

when genetically engineered mammalian cells were first shown to express biologically active hCG heterodimers (17). Since that time several laboratories have used mammalian cells to express glycoprotein hormone analogs which are capable of binding to receptors and inducing a biological function (10, 18-21). These analogs appear to be glycosylated similar to the naturally occurring hormones. While not important for hormone-receptor interaction, glycosylation of the hormones has been shown to be important for signal transduction in many species (22). In these procedures one introduces a "gene" that encodes the desired amino acid sequences into mammalian cells downstream of a promoter. Construction of these genes is a standard recombinant DNA procedure wherein the codons of the genes encoding the alpha or beta-subunits of the hormones are changed to encode amino acid residues of the desired analogs using the well established genetic code (23, 24). When these gene constructs are transfected into mammalian cells using standard protocols (23, 24), they direct the secretion of glycosylated hormone analogs into the culture media. These media can be assayed for the presence of immunological or biological activity (10, 21).

Using mammalian cell expression systems to make hormone analogs, Campbell et al. (10) engineered an analog which converted hCG from a hormone which bound to LH receptors to an analog which bound to FSH receptors and had only slightly higher affinity for LH receptors than FSH. Campbell et al. (10) were unsuccessful in obtaining analogs which had a high affinity for both LH and FSH receptors. As noted earlier, some naturally occurring analogs (i.e., eLH) can bind to both LH and FSH receptors and this property suggested that it should be possible to engineer human glycoprotein hormone analogs with the abilities to bind to both LH and FSH receptors. None of these analogs have been reported as yet.



One of the most interesting findings made from the studies of Campbell et al. (10) was that the region of hCG and hFSH which appeared to be important for receptor interaction was different from that which had been presumed to interact with the LH and FSH receptors based on the results of studies using synthetic peptides. Indeed, Campbell et al. (10) found that the amino acid residues which had been thought to control receptor binding specificity on the basis of studies using synthetic peptides (i.e., amino acids 38-57 of hCG and 32-51 of hFSH) did not convey receptor binding activity in the intact hormone, the form which had highest affinity for the receptors.

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#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts in graphic format the ability of hCG, hFSH, and the alpha, beta-heterodimeric polypeptide analogs of the present invention to inhibit binding of  $^{125}\text{I}$ -hCG to rat corpora luteal LH receptors.

Figure 2 depicts in graphic format the ability of hCG, hFSH, and the alpha, beta-heterodimeric polypeptide analogs of the present invention to inhibit binding of  $^{125}\text{I}$ -hFSH to bovine testes tissue homogenates.

Figure 3 illustrates the effects of hFSH, CF101-109, and CF94-117 on hFSH receptor dependent responses (left panels) and hCG and CF101-109 on hLH receptor dependent responses (right panel). The hFSH, CF101-109, and CF94-117 used in these assays was produced by C127 cell lines that were stably expressing hFSH or the analogs.

Figure 4 illustrates the effects of hFSH, hCG, and CF101-109 on a Y-1 adrenal cell line that was stably expressing the rat FSH receptor. Both hFSH and CF101-109

caused these cells to make progesterone (measured by radioimmunoassay) and round-up (detected by morphological observation). hCG was without effect on these assays. In other studies, hCG and CF101-109 caused rounding of Y-1 cells transfected with rat LH receptors. In the study shown here, Y-1F cells were added to a 24-well culture dish and grown until 60-70% confluent in F-12 medium containing 15% horse serum and 2.5% fetal bovine serum. Hormones were added at the amounts indicated in a total volume of 0.2 ml and incubation was continued for 4.5 hours at 37° C. The plates were examined for rounding at 30 minutes, one hour, and at the end of the study. Cells treated with hFSH and CF101-109 rounded. Cells treated with hCG did not round. At the end of the study, the cells and medium were frozen and saved for progesterone assay. The progesterone was determined by RIA using a commercial kit obtained from Leeco, Southfield Michigan.

Figure 5 (TOP PANEL) illustrates the abilities of hCG and a series of CF101-109 analogs to bind to rat LH receptors expressed in CHO cells. Figure 5 (BOTTOM PANEL) illustrates the abilities of hFSH and the same series of CF101-109 analogs to bind to human FSH receptors expressed in CHO cells. The concentrations of the analogs were determined using A113/B105 sandwich immunoassays. In both panels the solid lines are associated with the top 12 symbols. The dotted lines are associated with the lowest 5 symbols.

Figure 6A illustrates the abilities of CF101-109 analogs to bind to FSH receptors compared with that of hFSH. The values were obtained by comparing the abilities of the analogs and hFSH to inhibit binding of  $^{125}\text{I}$ -hFSH to CHO cells expressing FSH receptors. Figure 6B illustrates the abilities of CF101-109 analogs to bind to LH receptors compared with that of hCG. The values were obtained by comparing the abilities of the analogs and hCG to inhibit binding of  $^{125}\text{I}$ -hCG to CHO cells expressing LH receptors. Figure 6C illustrates the

relative LH and FSH potencies of the CF101-109 analogs. The values were obtained by dividing the potencies of the analogs relative to hCG by the potencies of the analogs relative to FSH.

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Figure 7 illustrates the abilities of hCG and a series of CF101-109 analogs to stimulate cyclic AMP formation from CHO cells stably expressing rat LH receptors (TOP PANEL) and the abilities of hFSH and the same series of CF101-109 analogs to stimulate cyclic AMP formation from CHO cells stably expressing human FSH (BOTTOM PANEL). The concentrations of the analogs were determined using A113/B105 sandwich immunoassays. In both panels the solid lines are associated with the top 12 symbols. The dotted lines are associated with the lowest 5 symbols.

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#### SUMMARY OF THE INVENTION

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The present invention pertains to an alpha, beta-heterodimeric polypeptide having binding affinity to vertebrate luteinizing hormone (LH) receptors and vertebrate follicle stimulating hormone (FSH) receptors comprising a glycoprotein hormone alpha-subunit polypeptide and a non-naturally occurring beta-subunit polypeptide, wherein the beta-subunit polypeptide is a chain of amino acids comprising the following four joined subsequences:

25

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(a) a first subsequence homologous to the amino acid sequence of residues 1-93 of the beta-subunit selected from the group consisting of human chorionic gonadotrophin (hCG), vertebrate luteinizing hormone (LH), vertebrate follicle stimulating hormone (FSH), and vertebrate thyroid stimulating hormone (TSH);

35

(b) a second subsequence homologous to the amino acid sequence of residues 94-97 of the beta-subunit selected from the group consisting of human chorionic gonadotrophin (hCG) and vertebrate luteinizing hormone (LH);

(c) a third subsequence homologous to the amino acid sequence of residues 98-100 of the beta-subunit selected from the group consisting of human chorionic gonadotrophin (hCG), vertebrate luteinizing hormone (LH), vertebrate follicle stimulating hormone (FSH), and vertebrate thyroid stimulating hormone (TSH); and

(d) a fourth subsequence homologous to the amino acid sequence of residues 101-110 of the beta-subunit of vertebrate follicle stimulating hormone.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to glycoprotein hormone analogs having altered receptor binding specificity and activity. Applicants have discovered that the residues important for receptor binding and specificity in glycoprotein hormones are located between Cys93 and Cys100 and Cys100 and Cys110 of hCG. These residues correspond to hFSH residues 87-94 and 94-104. A wide variety of alpha, beta-heterodimeric polypeptides can be made to alter the LH and FSH receptor binding activity and specificity of the polypeptides without disrupting their abilities to form alpha, beta-heterodimers or react with antibodies. The finding that interactions between regions of hCG beta-subunit amino acids 94-97 and 101-106 (corresponding to hFSH beta-subunit amino acids 88-91 and 95-100) play a dominant role in determining hormone-receptor binding specificity was unexpected because 1) Keutmann H.T. et al. (14), Santa coloma and Reichert (15), and Schneyer,

A.L., et al. (16) had shown that synthetic peptides containing beta-subunit amino acids from portions of the region 38-57 of hCG and 32-51 of hFSH were able to bind to LH and FSH receptors with low affinity and 2) Campbell et al. (10) had shown that residues 94-117 acted to cause an almost total shift in receptor binding specificity. The region containing amino acids 94-97 by itself did not influence receptor binding specificity and it was believed that modification of other residues in the 94-117 region of the molecule would also have an all-or-none effect on receptor binding specificity.

The alpha, beta-heterodimeric polypeptides of the present invention are "engineered" to alter the LH and FSH receptor binding activity and specificity of the polypeptides in vertebrates. Applicants have found that 1) the region of the hCG beta-subunit between 94-97 (i.e., "D" region) is most important for LH receptor binding activity and specificity, 2) the region of the hFSH beta-subunit between 100-106 (hCG numbering, i.e., "G" region) is most important for FSH binding activity and specificity and, 3) these regions ("D" and "G") of the beta-subunit are somewhat independent in activity. Substitution of a non-LH sequence in the "D" region will decrease binding of the polypeptide to the LH receptor. Substitution of a FSH sequence in the "G" region will increase binding of the polypeptide to the FSH receptor. Thus, it is possible to make modifications and substitutions in these regions to decrease the affinity of hCG for LH receptors and to dramatically increase the affinity of hCG for FSH receptors. The region of the beta-subunit between 1-93 and the choice of alpha-subunit does not appear to be important for LH and FSH binding activity and specificity. Hence analogs having a wide variety of activities can be made by altering the compositions of these two critical regions.

Generally, amino acid sequence variants will be substantially homologous with the relevant portion of the

alpha, beta-heterodimeric polypeptides as set forth in Tables 1 and 2. Substantially homologous means that greater than about 70% of the primary amino acid sequence of the candidate polypeptide corresponds to the sequence of the relevant portion of the alpha, beta-heterodimeric polypeptide when aligned in order to maximize the number of amino acids residue matches between the two polypeptides. Alignment to maximize matches of residues includes shifting the amino and/or carboxyl terminus, introducing gaps as required or deleting residues present as inserts in the candidate polypeptide, or both. For example, see Tables 1 and 2 where the glycoprotein hormones and the alpha, beta-heterodimeric polypeptides are aligned for maximum homology. Typically, amino acid sequence variants will be greater than about 90% homologous with the corresponding sequences shown for the proteins in Tables 1 and 2.

Variants that are not hormonally-active fall within the scope of this invention, and include polypeptides that may or may not be substantially homologous with a sequence described herein but which are 1) immunologically cross-reactive with antibodies raised against the counterpart polypeptide or 2) capable of competing with such counterpart polypeptides for cell surface receptor binding. Hormonally active variants are produced by the recombinant or organic synthetic preparation of fragments or by introduction of amino acid sequence variations so that the molecule no longer demonstrates hormonal activity as defined herein.

Immunological or receptor cross-reactivity means that the candidate polypeptide is capable of competitively inhibiting the binding of the hormonally-active analogue to polyclonal antisera raised against the hormonally-active analogue. Such antisera are prepared in a conventional manner by injecting goats or rabbits S.C. with the hormonally active analogue or derivative in

complete Freund's adjuvant, followed by booster intraperitoneal or S.C. injections in incomplete Freund's.

5 Variants that are not hormonally active but which are capable of cross-reacting with antisera to hormonally active polypeptides are useful a) as reagents in diagnostic assays for the analogues of their antibodies, b) when insolubilized in accord with known methods, as an agent for purifying anti-analogue anti-  
10 antibodies from anti-sera, and c) as an immunogen for raising antibodies to hormonally active analogues.

In accordance with the invention, the terminology "glycoprotein hormone" refers to human  
15 chorionic gonadotrophin (hCG), vertebrate luteinizing hormone (LH), vertebrate follicle stimulating hormone (FSH), and vertebrate thyroid stimulating hormone (TSH). The term "chimera" is used to designate a hormone analog which contains amino acid sequences derived from two or  
20 more different glycoprotein hormones.

In accord with the present invention, the alpha, beta-heterodimeric polypeptides comprise a glycoprotein hormone alpha-subunit polypeptide and a non-  
25 naturally occurring beta-subunit polypeptide. As set out above, the glycoprotein hormones share a common alpha-subunit. Substitution of the alpha-subunits of any one hormone for that of another does not significantly change the receptor binding properties of the new hormone. Accordingly, the alpha-subunit polypeptide in the present  
30 invention may be any vertebrate glycoprotein hormone alpha-subunit polypeptide. Nonlimiting examples of suitable alpha-subunit polypeptides include the alpha-subunits from human chorionic gonadotrophin (hCG),  
35 vertebrate luteinizing hormone (LH), vertebrate follicle stimulating hormone (FSH), and vertebrate thyroid stimulating hormone (TSH), and mixtures. In a preferred embodiment, the glycoprotein hormone alpha-subunit polypeptide may be selected from the group consisting of

human chorionic gonadotrophin (hCG) and vertebrate luteinizing hormone (LH). In a more preferred embodiment, the glycoprotein hormone alpha-subunit polypeptide is human chorionic gonadotrophin (hCG).  
5 Preferably the glycoprotein hormone alpha-subunit polypeptide is a human polypeptide.

The beta-subunit of the alpha, beta-heterodimeric polypeptide is a non-naturally occurring  
10 beta-subunit polypeptide which is a chain of amino acids comprising four joined subsequences. The four joined subsequences are as follows:

(a) a first subsequence homologous to the amino  
15 acid sequence of residues 1-93 of the beta-subunit selected from the group consisting of human chorionic gonadotrophin (hCG), vertebrate luteinizing hormone (LH), vertebrate follicle stimulating hormone (FSH), and vertebrate thyroid stimulating hormone (TSH);

20  
(b) a second subsequence homologous to the amino acid sequence of residues 94-97 of the beta-subunit selected from the group consisting of human chorionic gonadotrophin (hCG) and vertebrate luteinizing hormone  
25 (LH);

(c) a third subsequence homologous to the amino  
30 acid sequence of residues 98-100 of the beta-subunit selected from the group consisting of human chorionic gonadotrophin (hCG), vertebrate luteinizing hormone (LH), vertebrate follicle stimulating hormone (FSH), and vertebrate thyroid stimulating hormone (TSH);

(d) a fourth subsequence homologous to the  
35 amino acid sequence of residues 101-110 of the beta-subunit of vertebrate follicle stimulating hormone.

In one embodiment, the invention is directed to an alpha, beta-heterodimeric polypeptide having greater



binding affinity for vertebrate follicle stimulating hormone (FSH) receptors than for vertebrate luteinizing hormone (LH) receptors comprising a glycoprotein hormone alpha-subunit polypeptide and a non-naturally occurring beta-subunit polypeptide, wherein the beta-subunit polypeptide is a chain of amino acids comprising the following four joined subsequences:

(a) a first subsequence homologous to the amino acid sequence of residues 1-93 of the beta-subunit selected from the group consisting of human chorionic gonadotrophin (hCG), vertebrate luteinizing hormone (LH), vertebrate follicle stimulating hormone (FSH), and vertebrate thyroid stimulating hormone (TSH);

(b) a second subsequence comprising 4 amino acids for residues 94-97;

(c) a third subsequence comprising 3 amino acids for residues 98-100; and

(d) a fourth subsequence homologous to the amino acid sequence of residues 101-110 of the beta-subunit of vertebrate follicle stimulating hormone.

In another embodiment, the invention is directed to an alpha, beta-heterodimeric polypeptide having binding affinity to follicle stimulating hormone (FSH) receptors and luteinizing hormone (LH) receptors comprising a glycoprotein hormone alpha-subunit polypeptide and a non-naturally occurring beta-subunit polypeptide, wherein the beta-subunit polypeptide is a chimera comprised of amino acids 1-100 of any vertebrate glycoprotein hormone homologous to amino acids found in residues 1-100 of human chorionic gonadotropin and any 1-20 amino acids which binds LH receptors better than FSH receptors and has biological activity.

In a preferred embodiment, the alpha, beta-heterodimeric polypeptide is selected from the group consisting of "G", "DG'", "Q", "D", "GT", and "DGT" as set out in Tables 1 and 2. In a more preferred embodiment, the alpha, beta-heterodimeric polypeptide is "G".

Table 1 is a representation of the alpha, beta-heterodimeric polypeptides of the present invention using the one letter code and aligned in order to maximize the number of amino acids residue matches between the two polypeptides. Table 2 is a representation of the alpha, beta-heterodimeric polypeptides of the present invention using the three letter code. Abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe, G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

TABLE 1

AMINO ACID SEQUENCES OF THE BETA-SUBUNITS OF hCG, hLH, hFSH,  
AND THE alpha, beta-HETERODIMERIC POLYPEPTIDE ANALOGS

POLYPEPTIDE

ANALOG

AMINO ACID SEQUENCES OF THE BETA-SUBUNITS \*

10 20 30 40 50 60 70 80 90 100 110 120 130 140

SEQ ID NO: 1	hCG	SKEPLRPRCPINATLAVEKEGCPVCI	TWNTTICAGYCP	TNTRVLGGVLPALPQV	CHYRDVRFESIRLPGCP	GVNPNVSYAVALSQCCAL	CRRTTDCGGPKDHPLTCD	DRFQSSSSKAPPSLPS	SRLPGSDTPILPQ				
SEQ ID NO: 2	rhCG	SKEPLRPRCPINATLAVEKEGCPVCI	TWNTTICAGYCP	TNTRVLGGVLPALPQV	CHYRDVRFESIRLPGCP	GVNPNVSYAVALSQCCAL	CRRTTDCGGPKDHPLTCD	DRFQSSSSKAPPSLPS	SRLPGSDTPILPQ				
SEQ ID NO: 3	hFSH	NSCELNTITIAIEKEEC	FCISINITWCAGYCY	TRDLVYKDPARPKIQKT	CTFKELVYTVRVP	GCAHADS	LYTYPVALQCHGK	CDSDSTOCTVRGL	GPSYCSFGENKE				
SEQ ID NO: 4	rhFSH	NSCELNTITIAIEKEEC	FCISINITWCAGYCY	TRDLVYKDPARPKIQKT	CTFKELVYTVRVP	GCAHADS	LYTYPVALQCHGK	CDSDSTOCTVRGL	GPSYCSFGENKE				
SEQ ID NO: 5	eCG	SRGPLRPLCRPINATLAE	KEACPICITFTTIS	CAGYCP	THSHVRVMPAALPAIPQ	VCTYRELRFASIRLPGCP	PGVDPMVSFPVALSCHG	PCQIKTTDCGV	FRDQPLACAPQASSSSKOPPS	SQPLTSTSTPTPGASRSSHPLPIKTS			
SEQ ID NO: 6	hTSH	FCIPTETYNTHIERRE	CAYCLINTTICAGY	CHTRDINGKLELPKY	ALSQDVCTYRDFIYR	VEIPQCPLHVAPYFS	VPVALSCCKGKCDTDY	SDCIHEA	IKTNYCTKPQKSY				
SEQ ID NO: 7	G	SKEPLRPRCPINATLAVE	KEGCPVCI	TWNTTICAGYCP	TNTRVLGGVLPALPQV	CHYRDVRFESIRLPGCP	GVNPNVSYAVALSQCCAL	CRRTTDC	TVRGLGPSYCSFGE				
SEQ ID NO: 8	DG'	SKEPLRPRCPINATLAVE	KEGCPVCI	TWNTTICAGYCP	TNTRVLGGVLPALPQV	CHYRDVRFESIRLPGCP	GVNPNVSYAVALSQCCAL	CRRTTDC	TVRGLGPSYCSFGE				
SEQ ID NO: 9	Q	SKEPLRPRCPINATLAVE	KEGCPVCI	TWNTTICAGYCP	TNTRVLGGVLPALPQV	CHYRDVRFESIRLPGCP	GVNPNVSYAVALSQCCAL	CRRTTDC	TVRGLGPSYCSFGE				
SEQ ID NO: 10	D	SKEPLRPRCPINATLAVE	KEGCPVCI	TWNTTICAGYCP	TNTRVLGGVLPALPQV	CHYRDVRFESIRLPGCP	GVNPNVSYAVALSQCCAL	CRRTTDC	TVRGLGPSYCSFGE				
SEQ ID NO: 11	GT	SKEPLRPRCPINATLAVE	KEGCPVCI	TWNTTICAGYCP	TNTRVLGGVLPALPQV	CHYRDVRFESIRLPGCP	GVNPNVSYAVALSQCCAL	CRRTTDC	TVRGLGPSYCSFGE				
SEQ ID NO: 12	DGT	SKEPLRPRCPINATLAVE	KEGCPVCI	TWNTTICAGYCP	TNTRVLGGVLPALPQV	CHYRDVRFESIRLPGCP	GVNPNVSYAVALSQCCAL	CRRTTDC	TVRGLGPSYCSFGE				

\* NON-hCG DERIVED RESIDUES IN THE CHIMERAS ARE UNDERLINED

TABLE 2

AMINO ACID SEQUENCES OF THE beta-SUBUNITS OF hCG, hLH, hFSH,  
AND THE alpha, beta-HETERODIMERIC POLYPEPTIDE ANALOGS

5	hCG
10	SerLysGluProLeuArgProArgCysArgProIleAsnAlaThrLeuAlaValGluLysGluGlyCysProVal CysIleThrValAsnIleThrIleCysAlaGlyTyrCysProThrMetThrArgValLeuGlnGlyValLeuPro AlaLeuProGlnValValCysAsnTyrArgAspValArgPheGluSerIleArgLeuProGlyCysProArgGly ValAsnProValValSerTyrAlaValAlaLeuSerCysGlnCysAlaLeuCysArgArgSerThrThrAspCys GlyGlyProLysAspHisProLeuThrCysAspAspProArgPheGlnAspSerSerSerSerLysAlaProPro ProSerLeuProSerProSerArgLeuProGlyProSerAspThrProIleLeuProGln
15	hFSH
20	AsnSerCysGluLeuThrAsnIleThrIleAlaIleGluLysGluGluCysArgPheCysIleSerIleAsnIle ThrTrpCysAlaGlyTyrCysTyrThrArgAspLeuValTyrLysAspProAlaArgProLysIleGlnLysThr CysThrPheLysGluLeuValTyrGluThrValArgValProGlyCysAlaHisHisAlaAspSerLeuTyrThr TyrProValAlaLeuGlnCysHisCysGlyLysCysAspSerAspSerThrAspCysThrValArgGlyLeuGly ProSerTyrCysSerPheGlyGluMetLysGlu
	eCG
25	SerArgGlyProLeuArgProLeuCysArgProIleAsnAlaThrLeuAlaAlaGluLysGluAlaCysProIle CysIleThrPheThrThrSerIleCysAlaGlyTyrCysProSerMetValArgValMetProAlaAlaLeuPro AlaIleProGlnProValCysThrTyrArgGluLeuArgPheAlaSerIleArgLeuProGlyCysProProGly ValAspProMetValSerPheProValAlaLeuSerCysHisCysGlyProCysGlnIleLysThrThrAspCys GlyValPheArgAspGlnProLeuAlaCysAlaProGlnAlaSerSerSerSerLysAspProProSerGlnPro LeuThrSerThrSerThrProThrProGlyAlaSerArgArgSerSerHisProLeuProIleLysThrSer
30	hTSH
35	PheCysIleProThrGluTyrMetThrHisIleGluArgArgGluCysAlaTyrCysLeuThrIleAsnThrThr IleCysAlaGlyTyrCysMetThrArgAspIleAsnGlyLysLeuPheLeuProLysTyrAlaLeuSerGlnAsp ValCysThrTyrArgAspPheIleTyrArgThrValGluIleProGlnCysProLeuHisValAlaProTyrPhe SerTyrProValAlaLeuSerCysLysCysGlyLysCysAspThrAspTyrSerAspCysIleHisGluAlaIle LysThrAsnTyrCysThrLysProGlnLysSerTyr
40	Analog "G"
45	SerLysGluProLeuArgProArgCysArgProIleAsnAlaThrLeuAlaValGluLysGluGlyCysProVal CysIleThrValAsnIleThrIleCysAlaGlyTyrCysProThrMetThrArgValLeuGlnGlyValLeuPro AlaLeuProGlnValValCysAsnTyrArgAspValArgPheGluSerIleArgLeuProGlyCysProArgGly ValAsnProValValSerTyrAlaValAlaLeuSerCysGlnCysAlaLeuCysArgArgSerThrThrAspCys ThrValArgGlyLeuGlyProSerTyrCysAspAspProArg
	Analog "DG"
50	SerLysGluProLeuArgProArgCysArgProIleAsnAlaThrLeuAlaValGluLysGluGlyCysProVal CysIleThrValAsnIleThrIleCysAlaGlyTyrCysProThrMetThrArgValLeuGlnGlyValLeuPro AlaLeuProGlnValValCysAsnTyrArgAspValArgPheGluSerIleArgLeuProGlyCysProArgGly ValAsnProValValSerTyrAlaValAlaLeuSerCysGlnCysAlaLeuCysAspSerAspSerThrAspCys ThrValArgGlyLeuGlyProSerTyrCysSerPheGlyGlu

TABLE 2 (continued)

## Analog "Q"

5 SerLysGluProLeuArgProArgCysArgProIleAsnAlaThrLeuAlaValGluLysGluGlyCysProVal  
 CysIleThrValAsnIleThrIleCysAlaGlyTyrCysProThrMetThrArgValLeuGlnGlyValLeuPro  
 AlaLeuProGlnValValCysAsnTyrArgAspValArgPheGluSerIleArgLeuProGlyCysProArgGly  
 ValAsnProValValSerTyrAlaValAlaLeuSerCysGlnCysAlaLeuCysAspSerAspSerThrAspCys  
 10 GlyGlyProLysAspHisProSerTyrCysSerPheGlyGlu

## Analog "D"

15 SerLysGluProLeuArgProArgCysArgProIleAsnAlaThrLeuAlaValGluLysGluGlyCysProVal  
 CysIleThrValAsnIleThrIleCysAlaGlyTyrCysProThrMetThrArgValLeuGlnGlyValLeuPro  
 AlaLeuProGlnValValCysAsnTyrArgAspValArgPheGluSerIleArgLeuProGlyCysProArgGly  
 ValAsnProValValSerTyrAlaValAlaLeuSerCysGlnCysAlaLeuCysAspSerAspSerThrAspCys  
 GlyGlyProLysAspHisProLeuThrCysAspAspProArgPheGlnAspSerSerSerSerLysAlaProPro  
 20 ProSerLeuProSerProSerArgLeuProGlyProSerAspThrProIleLeuProGln

## Analog "GT"

25 SerLysGluProLeuArgProArgCysArgProIleAsnAlaThrLeuAlaValGluLysGluGlyCysProVal  
 CysIleThrValAsnIleThrIleCysAlaGlyTyrCysProThrMetThrArgValLeuGlnGlyValLeuPro  
 AlaLeuProGlnValValCysAsnTyrArgAspValArgPheGluSerIleArgLeuProGlyCysProArgGly  
 ValAsnProValValSerTyrAlaValAlaLeuSerCysGlnCysAlaLeuCysArgArgSerThrThrAspCys  
 IleHisGluAlaIleLysThrAsnTyrCysThrLysProGlnLysSerTyr

## Analog "DGT"

30 SerLysGluProLeuArgProArgCysArgProIleAsnAlaThrLeuAlaValGluLysGluGlyCysProVal  
 CysIleThrValAsnIleThrIleCysAlaGlyTyrCysProThrMetThrArgValLeuGlnGlyValLeuPro  
 AlaLeuProGlnValValCysAsnTyrArgAspValArgPheGluSerIleArgLeuProGlyCysProArgGly  
 ValAsnProValValSerTyrAlaValAlaLeuSerCysLysCysGlyLysCysAspThrAspTyrSerAspCys  
 35 IleHisGluAlaIleLysThrAsnTyrCysThrLysProGlnLysSerTyr

The activities of alpha, beta-heterodimeric polypeptide analogs "DG'," "Q," "D," "GT," and "DGT" are illustrated in Table 3, Figure 1, and Figure 2 along with hCG, hFSH, and eLH controls. The properties of some of these analogs have been described previously (10).

Table 3  
ACTIVITIES OF THE alpha, beta-HETERODIMERIC POLYPEPTIDE ANALOGS RELATIVE TO THAT OF hCG AND hFSH.

ANALOG	LH RECEPTORS	FSH RECEPTORS
rhCG	1.0	N.D.
rhFSH	N.D.	1.0
eCG	0.53	0.18
G	0.62	0.29
DG'	N.D.	0.32
Q	0.15	N.D.
D	0.065	N.D.
GT	0.33	N.D.
DGT	N.D.	N.D.

These values were obtained by dividing the concentration of analog required to inhibit  $^{125}\text{I}$ -hCG binding to rat luteal ovarian LH receptors or  $^{125}\text{I}$ -hFSH binding to bovine testes FSH receptors by 50% into that required for recombinant hCG and recombinant hFSH, respectively. The concentrations of the analogs were determined by sandwich immunoassay using antibodies B105 and A113. The term "N.D." means not determined because concentrations of analog were not employed high enough to detect 50% inhibition. This was because the amounts of hFSH needed to bind to LH Receptors are several orders of magnitude greater than that of hCG and vice versa the

amounts of hCG needed to bind to FSH receptors are several orders of magnitude greater than that of hFSH.

These results show that it is possible to  
5 control the ratio of FSH:LH activity by modulating residues corresponding to hCG amino acids 94-99 and 101-109, hFSH amino acids 88-93 and 95-103, or hTSH amino acids 89-94 and 96-104. These regions are termed "D" and "G", respectively (c.f., Table 1). In general,  
10 substitution of a non-LH sequence in the "D" region decreases binding of the polypeptide to the LH receptor while substitution of a FSH sequence in the "G" region increases binding of the polypeptide to the FSH receptor. Analogs containing both "D" and "G" from hCG bind to LH  
15 receptors like hCG (10). Analogs containing both "D" and "G" from hFSH bind to FSH receptors like FSH (10). Analogs containing "D" from hCG and "G" from hFSH bind well to both LH and FSH receptors (Table 3, Figure 1, and Figure 2). Analogs containing "D" from hFSH and "G" from  
20 hCG bind to LH receptors with considerably lower potency than hCG (10, Table 3, Figure 1, and Figure 2) and do not bind to FSH receptors. Analogs with "D" from hCG and "G" from TSH (i.e., "GT") bind to LH receptors but not to FSH receptors (Table 3, Figure 1, and Figure 2). Analogs  
25 with "D" and "G" from hTSH do not bind very well to either LH or FSH receptors (Table 3, Figure 1, and Figure 2).

These results show that it is possible to  
30 convert a human hormone into a molecule which binds to both LH and FSH receptors by using the "D" region from hCG and the "G" region from hFSH. Since replacement of the "D" region of hCG by residues from the "D" region of hFSH or hTSH dramatically lowers the ability of the  
35 analog to bind to LH receptors (i.e., compare hCG with analog "D," analog "G" with analog "DG'," and analog "GT" with analog "DGT"), it is clear that hFSH or hTSH substitutions in the "D" region which will make the analog less like hCG and will decrease its ability to

bind to LH receptors. If the "G" region in these analogs is derived from hFSH, they will bind well to FSH receptors (i.e., compare analog "G" with analog "DG'"). Thus, as the residues in the "D" region are changed from those of hCG or any other hormone which binds to LH receptors to those of hFSH or hTSH, the ratios of LH:FSH activities will decrease and the analog will appear to behave more like FSH.

While the "D" region appears to have the greatest influence on binding of the analogs to LH receptors, the "G" region appears to have the greatest influence on binding of the analogs to FSH receptors. Thus, analog "G" binds FSH receptors better than analog "GT." Similarly, analog "DG'" binds FSH receptors better than analog "D" (Table 3, Figure 1, and Figure 2). Thus, as the residues in the "G" region are changed from hFSH or any other hormone which binds to FSH receptors to those of hCG or hTSH, the molecule will lose its ability to bind to FSH receptors, the ratios of LH:FSH activities will increase, and the analog will appear to behave more like hCG or LH.

By systematically varying the composition of residues in the "D" and "G" region between those of hCG, hFSH, and hTSH, it is possible to create a hormone analog which has any desired ratio of LH:FSH activity. Other regions of the hormone do not appear to have significant influence on receptor binding specificity (10).

Certain data suggest that the minimum sizes of regions "D" and "G" may be smaller than the sizes indicated. Thus in region "D" there are only 4 amino acids which differ between hCG and hFSH. Of these amino acids, only three are highly non-conserved (i.e., RRS/DSD). In region "G" there are 8 amino acids different between hCG and hFSH, however, it is clear that only 6 of these amino acid residues play an important role in receptor binding specificity. Thus, analog "Q"



retains its ability to bind to LH receptors (Table 3, Figure 1, and Figure 2) even though it has two amino acid residues (i.e., 108 and 109) which are specifically found in the "G" region of hFSH, not hCG. On this basis, only  
5 6 residues are believed to be critical in the "G" region.

The compounds of the present invention while effective in the form of the free form may be formulated and administered in the form of the therapeutically or  
10 pharmaceutically acceptable acid addition salts for purposes of stability, convenience of crystallization, increased solubility and the like. These acid addition salts include inorganic acid salts such as hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, perchloric  
15 acid salts and the like; and organic acid salts such as acetic, trifluoroacetic, propionic, oxalic, hydroxyacetic, methoxyacetic, 2-hydroxypropanoic, 2-oxopropanoic, propanedioic, 2-hydroxy-butanedioic, benzoic, 2-hydroxybenzoic, 4-amino-2-hydroxybenzoic,  
20 3-phenyl-2-propenoic, alpha-hydroxybenzeneacetic, methanesulfonic, ethanesulfonic, benzenesulfonic, toluenesulfonic, cyclohexanesulfamic, succinic, tartaric, citric, maleic, fumaric acid salts and the like. The preferred acid addition salts are chloride, oxalate and  
25 citrate. These acid addition salts can be prepared by conventional methods, such as by treatment of the free base of the inventive compound with the appropriate acid.

The compounds of the present invention,  
30 prepared in the free form, can be combined with a pharmaceutically acceptable carrier to provide a pharmaceutical composition. Suitable carriers for the free bases include propylene glycol-alcohol-water, isotonic water, sterile water for injection (USP),  
35 emulphor<sup>TM</sup>-alcohol-water, cremophor-EL<sup>TM</sup> or other suitable carriers known to those skilled in the art.

The compounds of the present invention, prepared in the pharmaceutically acceptable acid addition

salt form, can also be combined with a pharmaceutically acceptable carrier to provide a pharmaceutical composition. Suitable carriers for the acid addition salts include isotonic water, sterile water for injection (USP), alone or in combination with other solubilizing agents such as ethanol, propylene glycol, or other conventional solubilizing agents known to those skilled in the art.

Of course, the type of carrier will vary depending upon the mode of administration desired for the pharmaceutical composition as is conventional in the art. A preferred carrier is an isotonic aqueous solution of the inventive compound.

The compounds of the present invention can be administered to mammals, e.g., animals or humans, in amounts effective to provide the desired therapeutic effect. Since the activity of the compounds and the degree of the desired therapeutic effect vary, the dosage level of the compound employed will also vary. The actual dosage administered will also be determined by such generally recognized factors as the body weight of the patient and the individual hypersensitiveness of the particular patient. Thus, the unit dosage for a particular patient (man) can be as low as about 0.00005 mg/kg, which the practitioner may titrate to the desired effect.

The compounds of the present invention can be administered parenterally, in the form of sterile solutions or suspensions, such as intravenously, intramuscularly or subcutaneously in the carriers previously described. The compounds may also be administered orally, in the form of pills, tablets, capsules, troches, and the like, as well as sublingually, rectally, or transcutaneously with a suitable pharmaceutically acceptable carrier for that particular mode of administration as is conventional in the art.

For parental therapeutic administration, the compounds of the present invention may be incorporated into a sterile solution or suspension. These preparations should contain at least about 0.1% of the inventive compound, by weight, but this amount may be varied to between about 0.1% and about 50% of the inventive compound, by weight of the parental composition. The exact amount of the inventive compound present in such compositions is such that a suitable dosage level will be obtained. Preferred compositions and preparations according to the present invention are prepared so that a paranteral dosage unit contains from between about 0.5 milligrams to about 100 milligrams of the inventive compound.

The sterile solutions or suspensions may also include the following adjuvants: a sterile diluent, such as water for injection, saline solution, fixed oils, polyethylene glycol, glycerine, propylene glycol, or other synthetic solvent; antibacterial agents, such as benzyl alcohol or methyl paraben; antioxidants, such as ascorbic acid or sodium metabisulfite; chelating agents, such as ethylenediaminetetraacetic acid (EDTA); buffers, such as acetates, citrates or phosphates; and agents for the adjustment of tonicity, such as sodium chloride or dextrose. The parental preparations may be enclosed in ampules, disposable syringes, or multiple dose vials made of glass or plastic.

The compounds of the present invention can also be administered orally. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of tablets, capsules, elixirs, suspensions, syrups, wafers, chewing gums and the like. These preparations should contain at least about 4% of the inventive compound, by weight, but this amount may be varied depending upon the particular dosage form from between about 4% to about 70% of the inventive

compound, by weight of the oral composition. The exact amount of the compound present in the composition is such that a suitable dosage will be obtained. Preferred compositions and preparations according to the present invention are prepared so that an oral dosage unit form contains from between about 5 to about 300 milligrams of the inventive compound.

The tablets, pills, capsules, troches and the like may also contain the following adjuvants: a binder, such as microcrystalline cellulose, gum tragacanth or gelatine; an excipient, such as starch or lactose; a disintegrating agent, such as alginic acid, Primogel, corn starch and the like; a lubricating agent, such as magnesium stearate or Sterotex; a gliding agent, such as colloidal silicon dioxide; a sweetening agent, such as sucrose or saccharin; and a flavoring agent, such as peppermint, methyl salicylate or orange flavoring. When the dosage form is a capsule, it may additionally contain a liquid carrier such as a fatty oil. Other dosage unit forms may contain other materials which modify the physical form of the dosage unit, such as enteric coatings. Thus tablets or pills may be coated with sugar, shellac, or other enteric coating agents. A syrup may contain, in addition to the above adjuvants, sucrose as a sweetening agent, preservatives, dyes, coloring agents and flavoring agents.

It is especially advantageous to formulate the pharmaceutical compositions in dosage unit forms for ease of administration and uniformity of dosage. The term dosage unit forms as used herein refers to physically discrete units suitable for use as a unitary dosage, each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the pharmaceutical carrier. Examples of such dosage unit forms are tablets (including scored or coated tablets), capsules, pills, powder packets, wafers, injectable solutions or suspensions,

teaspoonfuls, tablespoonfuls and the like, and segregated multiples thereof.

Throughout this application, various  
5 publications have been referenced. The disclosures in these publications are incorporated herein by reference in order to more fully describe the state of the art.

The present invention is further illustrated by  
10 the following examples which are not intended to limit the effective scope of the claims. All parts and percentages in the examples and throughout the specification and claims are by weight of the final composition unless otherwise specified.

15

#### EXAMPLES

##### Preparation of "GT"

20 pSVL $\beta'$  was prepared by subcloning of the XhoI-BamHI fragment of pKBM-hCGB' vector (10) into the XhoI-BamHI sites of pSVL (obtained from Pharmacia Co., Piscataway, NJ). The approximately 3.6-3.7Kbp PvuII-SstI fragment from pSVL-hCGB' was ligated with the synthetic  
25 oligonucleotide pair:

C Q C A L C R R S T T D C I H E A I K T N Y C T K P Q K S Y \*  
5'-

CTGTCAATGTGCACTCTGCCGCAGATCTACCACTGACTGCATACATGAAGCCATCAAGACAACTACTGCACCAAACCTCAGAAGTCCTACTGAA  
30 GGCAGGTGAGCT-3'

and

3'-

GACAGTTACACGTGAGACGGCGTCTAGATGGTGACTGACGTATGTACTTCGGTAGTTCTGTTTGATGACGTGGTTGGAGTCTTCAGGATGACTT  
35 CCGTCCAC-5'.

An aliquot of the ligation mixture was taken and used to transform DH5-alpha strain E. coli. (obtained from Bethesda Research Laboratories, Gaithersburg, MD). Plasmid DNAs from ampicillin-resistant DH5-alpha clones

were screened by digestion with BglII (which is unique to vectors containing the cassette) and EcoRI (which cuts in the vector). Positive clones were identified by the presence of two fragments (approximately 0.8Kbp and 2.9Kbp). The sequence in the coding region of one of these plasmids, which lacked most of the beta-subunit cDNA due to excision of the PvuII fragment, was confirmed by dideoxysequencing as described (10). The remainder of the beta-subunit cDNA (encoding hCGB amino acids 1-87) was restored by ligation of the 2.3Kbp PvuI-PvuII fragment of this vector and the 2.9Kbp PvuI-PvuII fragment from pSVL-hCG-beta'. The ligation mixture was used to transform DH5-alpha strain E. coli. and ampicillin resistant clones were obtained. Miniprep plasmid DNA from these clones were digested with EcoRI and BglII, and DNA from positive clones exhibited fragments of approximately 2.5Kbp and 2.9Kbp. After the DNA was subjected to a dideoxy sequencing procedure to confirm that it encoded "GT" (Table 1), the plasmid DNA was then cotransfected into COS-7 cells (obtained from the American Type Culture Collection) along with pSVL-hCG-alpha, a pSVL-based plasmid encoding the human glycoprotein hormone alpha-subunit (10, 21), using a DEAE-dextran procedure (10). Beginning in 1-2 days and for a few days thereafter, the COS-7 cells produced significant amounts of the free subunits and the heterodimer. These were present in the culture media and heterodimer was detected using sandwich immunoassays employing monoclonal antibodies A113 and B105 (10). The protein was concentrated by ultrafiltration and monitored for its abilities to bind to LH and FSH receptors by radioligand receptor assays using  $^{125}\text{I}$ -hCG and  $^{125}\text{I}$ -hFSH as tracers and rat ovarian corpora lutea and bovine testes as sources of LH and FSH receptors as described (10).

## Preparation of "G"

We have found that an alpha, beta-heterodimer composed of the alpha-subunit of hCG and an hCG/hFSH beta-subunit chimera termed "G" having the amino acid sequence illustrated in Table 1 has high affinity for LH and FSH receptors as shown by its ability to compete with radiolabeled hCG and/or hFSH for binding to these receptors (Table 3, Figures 1 and 2). This analog can be prepared in a variety of methods well-known to one versed in the art of molecular biology, one of which is described here. The cDNA for analog "GT" was digested with BglII and SstI and the 5.2-5.3Kbp fragment was ligated with the oligonucleotides:

R S T T D C T V R G L G P S Y C D D P R \*

5'-GATCTACCACTGACTGCACCGTGAGAGGCCCTCGGGCCCTCTTACTGCGATGACCCGCGGTAGAGCT-3'

and

3'-ATGGTGACTGACGTGGCACTCTCCGGAGCCCGGGAATGACGCTACTGGGCGCCATC-5'

using standard methods (23, 24). The ligation mixture was used to transform competent DH5-alpha strain E. coli. (23, 24). Transformed cells were selected by their abilities to grow on agar plates containing ampicillin. Ampicillin resistant colonies were chosen and plasmid minipreparations were made by the boiling lysis method (23, 24). The plasmid DNA was then tested for the presence of HindIII-ApaI endonuclease restriction sites. Plasmid DNA having the desired sequences was cleaved into three fragments (approximately 0.8Kbp, 1.1Kbp, and 3.4Kbp). After the DNA was subjected to a dideoxy sequencing procedure to confirm that it encoded "G" (Table 1), the plasmid DNA was then cotransfected into COS-7 cells (obtained from the American Type Culture Collection) along with pSVL-hCG-alpha, a pSVL-based plasmid encoding the alpha-subunit (10, 21), using a DEAE-dextran procedure (10, 21, 23, 24). Beginning in 1-2 days and for a few days thereafter, the COS-7 cells produced significant amounts of the free subunits and the

heterodimer. These were present in the culture media and heter dimer was detected using sandwich immunoassays employing monoclonal antibodies A113 and B105 (10). The protein was concentrated by ultrafiltration and monitored  
5 for its abilities to bind to LH and FSH receptors by radioligand receptor assays using  $^{125}\text{I}$ -hCG and  $^{125}\text{I}$ -hFSH as tracers and rat ovarian corpora lutea and bovine testes as sources of LH and FSH receptors as described (10).

10

#### Preparation of "D" and "DG'" and "Q"

Preparations of analogs "D" and "DG'" have been described previously (10) and are the same as those of  
15 analogs CF94-97 and CF94-114 in that report, respectively. Analog "Q" was prepared from the expression vector encoding analogs "D" and CF108-114 (10) by digesting them with PpuMI, separating the fragments on agarose gels, and ligating the large fragment obtained  
20 from CF108-114 with the small fragment obtained from "D." The resulting plasmid was then cotransfected into COS-7 cells along with pSVL-hCG-alpha and the media assayed for the presence of the analogs using an A113-B105 sandwich immunoassay as described (10).

25

#### Preparation of "DGT"

Plasmid pSVL-hCG $\beta$ ' was sequentially digested with SstI and PvuII and the 3.6Kbp fragment was ligated  
30 with the synthetic DNA cassette formed by annealing the following oligonucleotides:



C K C G K C N T D Y S D C I H E A I K T N T C T K P Q K S Y \*  
5'-  
CTGTAAGTGTGGCAAGTGCAATACTGACTACAGTGACTGTATACATGAAGCCATCAAGACAAACTACTGCACCAAACCTCAGAAGTCCTACTGAA  
5 GGCAGGTGAGCT-3'  
and  
3'-  
GACATTCACACCGTTCACGTTATGACTGATGTCACTGACATATGTACTTCGGTAGTTCTGTTTGATGACGTGGTTGGAGTCTTCAGGATGACTT  
CCGTCCAC-5'.

10

The ligation mixture was used to transform DH5-  
alpha E. coli. and miniprep plasmid DNA obtained from  
ampicillin resistant colonies was screened for the  
presence of an approximately 0.6Kbp fragment released by  
15 digestion with AccI. After DNA sequencing was performed  
to confirm that the construct encoded the desired  
sequence, it was cut with PvuII and ligated with the  
1.6Kbp fragment of pSVL-hCGB'. The ligation product was  
transformed into DH5-alpha strain E. coli. and positive  
20 clones were selected. Plasmid DNA was prepared by  
boiling lysis and digested with EcoNI and XhoI. DNA  
which had the insert in the correct orientation produced  
fragments approximately 2.6Kbp, 1.7Kbp, 0.5Kbp, 0.25Kbp,  
and 0.15Kbp. The plasmid DNA was then cotransfected into  
25 COS-7 cells (obtained from the American Type Culture  
Collection) along with pSVL-hCG-alpha, a pSVL-based  
plasmid encoding the alpha-subunit (10, 21), using a  
DEAE-dextran procedure (10, 21, 23, 24). Beginning in 1-  
2 days and for a few days thereafter, the COS-7 cells  
30 produced significant amounts of the free subunits and the  
heterodimer. These were present in the culture media and  
heterodimer was detected using sandwich immunoassays  
employing monoclonal antibodies A113 and B105 (10). The  
concentration of the protein was concentrated by  
35 ultrafiltration and monitored for its abilities to bind  
to LH and FSH receptors by radioligand receptor assays  
using <sup>125</sup>I-hCG and <sup>125</sup>I-hFSH as tracers and rat ovarian  
corpora lutea and bovine testes as sources of LH and FSH  
receptors as described (10).

The procedures set out below produce the alpha, beta-heterodimeric polypeptides in a transient fashion. As is well known in the art, vectors other than pSVL can be chosen which will enable the protein to be expressed in a stable fashion in larger quantities. The DNA sequences described above can be excised from pSVL and then subcloned into any other expression vector. Cell types other than COS-7 cells can be used to express the analogs. These include virtually any eucaryotic cell for which an expression vector is known or can be devised and include mammalian cells, insect cells, yeast cells, fungal cells, and plant cells.

The abilities of the analogs to inhibit binding of  $^{125}\text{I}$ -hCG to rat corpora luteal LH receptors was performed by procedures similar to those described previously. Immature rats were given injections of pregnant mares serum gonadotropin and hCG to induce the formation of corpora lutea. The ovaries were removed from the animals, homogenized, and stored at a concentration of approximately 5mg homogenate tissue in 100 ul buffer (25).

The abilities of the analogs to inhibit binding of  $^{125}\text{I}$ -hFSH to bovine testes tissue homogenates was performed by procedures similar to those described previously (10).

Figures 1 and 2 depict in graphic format the ability of hCG, hFSH, and the alpha, beta-heterodimeric polypeptide analogs of the present invention to inhibit binding of  $^{125}\text{I}$ -hCG to rat corpora luteal LH receptors and binding of  $^{125}\text{I}$ -hFSH to bovine testes tissue homogenates, respectively.

To obtain additional evidence for our observation that the determinants of LH and FSH receptor binding specificity are independent, we prepared a series of

analogs of hCG which had a wide range of LH/FSH activities. We started with the analog that had approximately equal LH and FSH binding activities (this analog has been called a variety of names including "G" and "CF101-109"). This analog contains hFSH beta-subunit residues 95-103 for hCG beta-subunit residues 101-109 in an hCG beta-subunit analog which is truncated after residue 114. The sequence of this analog is set out below. The residues derived from the hFSH beta-subunit are underlined. The leader sequence is omitted.

```

          9      23 26      34 38      57      72
SKEPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLGGVLPALPQVVCNYRDVRFESIRLPGC
          88 93      100      110
15 PRGVNPPVSYAVALSCQCALCRRSTTDCTVRGLGPSYCDOPR

```

To facilitate making analogs in the 94-97 region, we made intermediate analogs MB135 and MB140. These were modified to give the desired analogs using cassette mutagenesis with short synthetic DNA oligos (i.e., 18-mers).

ANALOG MB135: (Converts G<sup>102</sup>/V in hCG beta-subunit residues 1-114.) This analog was made after considering the sequences of all mammalian glycoprotein hormones with LH and FSH activities. One of these, equine LH, has the ability to bind to rat LH and FSH receptors. Equine LH is very similar to hCG except in the region between 101-109 (26). One residue which might be critical in this region is the valine at 102. Therefore, we changed the glycine found in hCG at position 102 to valine.

We synthesized oligos 435 and 436 having the following sequences:

```

435: 5'-GTGGCTCTCAGCTGTCAATGCCGCTCTGCCGAGATCTACCACTGACTGCGGGGTCCCTAAGGACCAC-3', and
436: 3'-TGACTGACGCCCCAGGGATTCTCGTGGGGAACTGGACACTACTGGGGGCGATTCTCGAGCCTAGGCACACC-5'.

```

These were annealed and filled-in using Taq polymerase to give a product which encodes amino acids homologous to those of hCG *beta*-subunit between 84-114 except at residue 102 and introduces the BssHII restriction site in the DNA as shown:

```

      88      93      100      110
V A L S C Q C A L C R R S T T D C G V P K D H P L T C D D P R *
GTGGCTCTCAGCTGTCAATGCGCGCTCTGCCGAGATCTACCACTGACTGCGGGGTCCCTAAGGACCACCCCTTGACCTGTCATGACCCCCGCTA
10  AGAGCTCGGATCCGTGAGG
CACCAGAGATCGACAGTTACGCGCGAGACGGCGTCTAGATGCTGACTGACGCCCCAGGGATTCTGGTGGGSAACTGGACACTACTGGGGGCGAT
TCTCGAGCCTAGGCACACC
      CAG^CTG      G^CGCGC      A^GATCT      GACNNNN^NNGTC(DrdI)
GAGCT^CGG^ATCC
15  PvuII      BssHII      BglII      PG^GWCCY(PpuMI)
(SacI-BamHI)
CCTNAGG(MstII)

```

(In this diagram, the sequences derived from the original oligos are underlined. The locations of restriction sites that were created are also noted.) The reaction product was subjected to endonuclease digestion with PvuII and SacI, purified on an agarose gel, and cloned into the PvuII-SacI sites of pSVL-hCG-*beta*' to give pSVL-MB135. [pSVL-hCG-*beta*' was made by ligating the small XhoI-BamHI fragment from pKBM-hCG-*beta*' into the XhoI-BamHI sites of pSVL (27). The construct was sequenced using standard dideoxy double strand sequencing methods. pSVL-MB135 was then co-transfected with pSVL-hCG-*alpha* (27) into COS-7 cells. We concentrated the media by placing them in a dialysis bag and then surrounding the dialysis bag with Aquacide II (Calbiochem) overnight at 4° C. The concentrated media were dialyzed against phosphate buffered isotonic saline (PBS). The amount of dimer was measured using a sandwich immunoassay procedure as described (28) except that antibody A113 (*alpha*-subunit specific) and radioiodinated antibody B105 (*beta*-subunit specific) were used as the capture and detection reagents, respectively. This

analog formed dimer and bound to LH receptors and stimulated cyclic AMP formation. It had low ability to bind to FSH receptors.

5                   ANALOG MB140: This analog was made from pSVL-MB135 and was engineered to have hFSH beta-subunit residues between 101-109. In addition, coding sequences for Aspartic acid and Leucine were added and that for Arginine 94 was eliminated. The latter change added a  
10                   BspMI site. The large fragment of BssHI/BspMI digestion will be referred to as pSVL-MB140\*. This fragment lacked coding sequences corresponding to residues 92, 93, 94, 95, and 96 of the hCG beta-subunit. Thus, we could add any coding sequence to this region by ligating pSVL-MB140\* with any synthetic DNA cassette. The residues  
15                   which were most studied corresponded to those for residues 94, 95, and 96 of hCG beta-subunit.

                  Oligos 438 and 439 used to make analog MB140  
20                   were made and had the sequences:

439: 5'-CTGTCAATGCGCGCTCTGCGACCTGCGAAGTACTACCGACTGCACTGTCCGCGGTCTTGGGCC-3' and

438: 3'-CGTGACAGGCGCCAGAACC CGGAGAATAACACTACTAGGGGCGATTCTCGAGCCTAGGCACACC-5'.

25                   These were annealed, extended with Tag polymerase, digested with BssHII and SacI, and then cloned into the BssHII and SacI sites of pSVL-MB135 by standard methods to give pSVL-MB140. The resulting vector had the following coding sequence for amino acids  
30                   84-114:

88                      93                      101                      111  
 V A L S C Q C A L C D L R S T T D C T V R G L G P S Y C D D P R  
 \*  
 5    GTGGCTCTCAGCTGTCAATGCGCGCTCTGCGACCTGCGAAGTACTACCGACTGCACTGTCCGCGGTCTTGGGCCCTTATTGTGATGATCCTAG  
     ATAAGAGCTCGGATCCGTGAGG  
     CACCGAGAGTCGACAGTTACGCGCGAGACGCTGGACGCTTCATGATGGCTGACGTGACAGGCCCGCAGAACCCGGGAGAATAACCACTACTAGGGGC  
     GATTCTCGAGCCTAGGCACACC  
                  CAG<sup>^</sup>CTG            G<sup>^</sup>CGCGC            ACCTGC(BspHI)            GACNNNN<sup>^</sup>NNGTC(DrdI)            GGGCCC(ApaI)  
 10    GAGCT<sup>^</sup>CGG<sup>^</sup>ATCC  
                  PvuII            BssHII                            AGTACT(ScaI)                            CCGC<sup>^</sup>GG(SstII)  
     (SacI-BamHI)

Note, as before, the sequence derived from the  
 15    primers is underlined and several restriction sites are  
     identified. pSVL-MB140 was expressed in COS-7 cells  
     along with pSVL-hCG-alpha. This analog had limited  
     ability to form dimer with the alpha-subunit and was  
     nearly inactive in most biological assays. The analog  
 20    was readily detected in a beta-subunit specific sandwich  
     assay employing antibodies B201 and radiolabeled B105 as  
     capture and detection antibodies, respectively (Table 5).  
     This indicated that it probably folded similarly to the  
     beta-subunit.

25    ANALOG MB144: (This changes the sequence DLRST  
     of ANALOG MB140 to DRST).

30    Synthetic oligos 440 and 441 having the  
     sequences:

440                      5'-CGCGTTGTGTGACAGATC-3' and  
 441                      3'-AACACACTGTCTAGATGA-5'

35    were ligated into pSVL-MB140\* by standard methods to give  
     pSVL-MB144:

88            93                            100  
           C A L C D R S T T D C  
           TGC GCGTTGTGTGACAGATCTACTACCGACTGC  
 5            ACGCGCAACACACTGTCTAGATGATGGCTGACG  
                                  AGATCT  
                                  BglIII

10            A BglIII site was created and the ScaI and  
           BssHII sites were destroyed. The change in sites was  
           used for screening of the DNA construct during cloning.  
           After its structure had been confirmed by DNA sequencing,  
           pSVL-MB144 was co-transfected into COS-7 cells with pSVL-  
           hCG- $\alpha$ . Media were concentrated and found to contain  
 15             $\alpha$ ,  $\beta$ -heterodimer using the sandwich assay. This  
           analog bound to LH and FSH receptors and stimulated  
           cyclic AMP accumulation (Figures 5 and 7). The ratio of  
           LH to FSH activity is described in Figure 4c.

20            ANALOG MB145: (This changes the sequence DLRST  
           of ANALOG MB140 to NRST).

          Synthetic oligos 442 and 443 having the  
           sequences:

25            442                            5'-CGCGTTGTGTAACAGATC-3' and  
           443                            3'-AACACATTGTCTAGATGA-5'

          were ligated into pSVL-MB140\* to give pSVL-MB145:

30    93                            100  
     C A L C N R S T T D C  
     TGC GCGTTGTGTAACAGATCTACTACCGACTGC  
     ACGCGCAACACATTGTCTAGATGATGGCTGACG  
 35    AGATCT  
     BglIII

          A BglIII site was created and the ScaI and  
           BssHII sites of pSVL-MB140\* were destroyed. The change

in sites was used for screening of the DNA construct during cloning. After its structure had been confirmed by DNA sequencing, pSVL-MB145 was co-transfected into COS-7 cells with pSVL-hCG-alpha. Media were concentrated and found to contain alpha, beta-heterodimer using the sandwich assay. This analog bound to LH and FSH receptors and stimulated cyclic AMP accumulation (Figures 5 and 7). The LH:FSH activity ratio is described in Figure 4c.

ANALOG MB146: (This changes the sequence DLRST of ANALOG MB140 to RSST).

Synthetic oligos 444 and 445 having the sequences:

444 5'-CGCGTTGTGTAGATCTTC-3' and  
445 3'-AACACATCTAGAAGATGA-5'

were ligated into pSVL-MB140\* by standard methods to give pSVL-MB146:

88 93 100  
C A L C R S S T T D C  
TGCGCGTTGTGTAGATCTTCTACTACCGACTGC  
ACGCGCAACACATCTAGAAGATGATGGCTGACG  
AGATCT  
BglIII

A BglIII site was created and the ScaI and BssHII sites were destroyed. The change in sites was used for screening of the DNA construct during cloning. After its structure had been confirmed by DNA sequencing, pSVL-MB146 was co-transfected into COS-7 cells with pSVL-hCG-alpha. Media were concentrated and found to contain alpha, beta-heterodimer using the sandwich assay. This analog bound to LH and FSH receptors and stimulated cyclic AMP accumulation (Figures 5 and 7). The ratio of LH to FSH activity is described in Figure 4c.



ANALOG MB147: (This changes the sequence DLRST of ANALOG MB140 to RSDT).

5                    Synthetic oligos 447 and 448 having the sequences:

447                    5'-CGCGTTGTGTAGATCTGA-3' and  
448                    3'-AACACATCTAGACTATGA-5'

10

were ligated into pSVL-MB140\* by standard methods to give pSVL-MB147:

15                    93                    100  
                  C A L C R S D T T D C  
                  TGCGCGTTGTGTAGATCTGATACTACCGACTGC  
                  ACGCGCAACACATCTAGACTATGATGGCTGACG  
                  AGATCT  
                  BglII

20

A BglII site was created and the ScaI and BssHII sites were destroyed. The change in sites was used for screening of the DNA construct during cloning. After its structure had been confirmed by DNA sequencing, pSVL-MB147 was co-transfected into COS-7 cells with pSVL-hCG-alpha. Media were concentrated and found to contain alpha, beta-heterodimer using the sandwich assay. This analog bound to LH and FSH receptors and stimulated cyclic AMP accumulation (Figures 5 and 7). The ratio of LH to FSH activity is described in Figure 4c.

30

ANALOG MB148: (This changes the sequence DLRST of ANALOG MB140 to RSNT).

35                    Synthetic oligos 449 and 450 having the sequences:

449                    5'-CGCGTTGTGTAGATCTAA-3' and  
450                    3'-AACACATCTAGATTATGA-5'

were ligated into pSVL-MB140\* by standard methods to give pSVL-MB148:

```

5              93              100
              C A L C R S N T T D C
              T G C G C G T T G T G T A G A T C T A A T A C T A C C G A C T G C
              A C G C G C A A C A C A T C T A G A T T A T G A T G G C T G A C G
              A G A T C T
10              B g l I I I

```

A BglIII site was created and the ScaI and BssHII sites were destroyed. The change in sites was used for screening of the DNA construct during cloning. After its structure had been confirmed by DNA sequencing, pSVL-MB148 was co-transfected into COS-7 cells with pSVL-hCG-*alpha*. Media were concentrated and found to contain *alpha*, *beta*-heterodimer using the sandwich assay. This analog bound to LH and FSH receptors and stimulated cyclic AMP accumulation (Figures 5 and 7). The ratio of LH to FSH activity is described in Figure 4c.

ANALOG MB149: (This changes the sequence DLRST of ANALOG MB140 to QRST).

Synthetic oligos 451 and 452 having the sequences:

```

451              5'-CGCGTTGTGTCAGAGATC-3' and
30  452              3'-AACACAGTCTCTAGATGA-5'

```

were ligated into pSVL-MB140\* by standard methods to give pSVL-MB149:

93 100  
 C A L C Q R S T T D C  
 TCGCGCTTGTGTCAGAGATCTACTACCGACTGC  
 5 ACGCGCAACACAGTCTCTAGATGATGGCTGACG  
 AGATCT  
 BglII

10 A BglII site was created and the ScaI and  
 BssHII sites were destroyed. The change in sites was  
 used for screening of the DNA construct during cloning.  
 After its structure had been confirmed by DNA sequencing,  
 pSVL-MB149 was co-transfected into COS-7 cells with pSVL-  
 hCG-*alpha*. Media were concentrated and found to contain  
 15 *alpha*, beta-heterodimer using the sandwich assay. This  
 analog bound to LH and FSH receptors and stimulated  
 cyclic AMP accumulation (Figures 5 and 7). The ratio of  
 LH to FSH activity is described in Figure 4c.

20 ANALOG MB150: (This changes the sequence DLRST  
 of ANALOG MB140 to RQST).

Synthetic oligos 514 and 515 having the  
 sequences:

25 514 5'-CGCGCTGTGTCGACAGAG-3' and  
 515 3'-GACACAGCTGTCTCATGA-5'

30 were ligated into pSVL-MB140\* by standard methods to give  
 pSVL-MB150:

93 100  
 C A L C R Q S T T D C  
 TCGCGCTGTGTCGACAGAGTACTACCGACTGC  
 35 ACGCGGACACAGCTGTCTCATGATGGCTGACG  
 G^CGCGC G^TCGAC A^GTACT  
 BssHIII SalI ScaI

Note, the ScaI and BssHII sites were not destroyed and a SalI site was added for screening. After its structure had been confirmed by DNA sequencing, pSVL-MB150 was co-transfected into COS-7 cells with pSVL-hCG-  
5 *alpha*. Media were concentrated and found to contain *alpha*, *beta*-heterodimer using the sandwich assay. This analog bound to LH and FSH receptors and stimulated cyclic AMP accumulation (Figures 5 and 7). The LH:FSH activity ratio is described in Figure 4c.

10

ANALOG MB151: (This changes the sequence DLRST of ANALOG MB140 to DSRT).

15

Synthetic oligos 455 and 456 having the sequences:

455 5'-CGCGTTGTGTGATTCTCG-3' and  
456 3'-AACACACTAAGAGCATGA-5'

20 were ligated into pSVL-MB140\* by standard methods to give pSVL-MB151:

93 100  
C A L C D S R T T D C  
25 TGCGCGTTGTGTGATTCTCGTACTACCGACTGC  
ACGCGCAACACACTAAGAGCATGATGGCTGACG

The ScaI and BssHII sites were destroyed and this information was used for screening. After its  
30 structure had been confirmed by DNA sequencing, pSVL-MB151 was co-transfected into COS-7 cells with pSVL-hCG-*alpha*. Media were concentrated and found to contain *alpha*, *beta*-heterodimer using the sandwich assay. This analog bound to LH and FSH receptors and stimulated  
35 cyclic AMP accumulation (Figures 5 and 7). The ratio of LH to FSH activity is described in Figure 4c.

ANALOG MB152: (This changes the sequence DLRST of ANALOG MB140 to DSAT).

Synthetic oligos 457 and 458 having the sequences:

5     457                    5'-CGCGTTGTGTGATTCTGC-3' and  
      458                    3'-AACACACTAAGACGATGA-5'

were ligated into pSVL-MB140\* by standard methods to give pSVL-MB152:

10

                          93                                100  
                          C A L C D S A T T D C  
                          TCGCGTTGTGTGATTCTGCTACTACCGACTGC  
                          ACGCGCAACACACTAAGACGATGATGGCTGACG

15

The ScaI and BssHII sites were destroyed and this information was used for screening. After its structure had been confirmed by DNA sequencing, pSVL-MB152 was co-transfected into COS-7 cells with pSVL-hCG-  
20     *alpha*. Media were concentrated and found to contain *alpha*, *beta*-heterodimer using the sandwich assay. This analog bound to LH and FSH receptors and stimulated cyclic AMP accumulation (Figures 5 and 7). The ratio of LH to FSH activity is described in Figure 4c.

25

ANALOG MB153: (This changes the sequence DLRST of ANALOG MB140 to DRDT).

30     Synthetic oligos 459 and 460 having the sequences:

      459                    5'-CGCGTTGTGTGATAGAGA-3' and  
      460                    3'-AACACACTATCTCTATGA-5'

35     were ligated into pSVL-MB140\* by standard methods to give pSVL-MB153:

5  
93 100  
C A L C D R D T T D C  
TGCGCGTTGTGTGATAGAGATACTACCGACTGC  
ACGCGCAACACACTATCTCTATGATGGCTGACG

10 The ScaI and BssHII sites were destroyed and this information was used for screening. After its structure had been confirmed by DNA sequencing, pSVL-MB153 was co-transfected into COS-7 cells with pSVL-hCG-alpha. Media were concentrated and found to contain alpha, beta-heterodimer using the sandwich assay. This analog bound to LH and FSH receptors and stimulated cyclic AMP accumulation (Figures 5 and 7). The ratio of  
15 LH to FSH activity is described in Figure 4c.

ANALOG MB154: (This changes the sequence DLRST of ANALOG MB140 to ASDT).

20 Synthetic oligos 461 and 462 having the sequences:

461 5'-CGCGTTGTGTGCTAGCGA-3' and  
462 3'-AACACACGATCGCTATGA-5'

25 were ligated into pSVL-MB140\* by standard methods to give pSVL-MB154:

30 93 100  
C A L C A S D T T D C  
TGCGCGTTGTGTGCTAGCGATACTACCGACTGC  
ACGCGCAACACACGATCGCTATGATGGCTGACG  
G^CTAGC  
NheI

35 The ScaI and BssHII sites were destroyed and a NheI site was created. This information was used for screening. After its structure had been confirmed by DNA sequencing, pSVL-MB154 was co-transfected into COS-7

cells with pSVL-hCG-*alpha*. Media were concentrated and found to contain *alpha*, *beta*-heterodimer using the sandwich assay. This analog bound to LH and FSH receptors and stimulated cyclic AMP accumulation (Figures 5 and 7). The ratio of LH to FSH activity is described in Figure 4c.

ANALOG MB155: (This changes the sequence DLRST of ANALOG MB140 to QIST).

10

Synthetic oligos 463 and 464 having the sequences:

463 5'-CGCGTTGTGTCAGATCTC-3' and  
15 464 3'-AACACAGTCTAGAGATGA-5'

were ligated into pSVL-MB140\* by standard methods to give pSVL-MB155:

20

93 100  
C A L C Q I S T T D C  
TGCGCGTTGTGTCAGATCTCTACTACCGACTGC  
ACGCGCAACACAGTCTAGAGATGATGGCTGACG

25

A<sup>^</sup>GATCT

BglII

A BglII site was created and the ScaI and BssHII sites were destroyed. The change in sites was used for screening of the DNA construct during cloning. After its structure had been confirmed by DNA sequencing, pSVL-MB155 was co-transfected into COS-7 cells with pSVL-hCG-*alpha*. Media were concentrated and found to contain *alpha*, *beta*-heterodimer using the sandwich assay. This analog bound to LH and FSH receptors and stimulated cyclic AMP accumulation (Figures 5 and 7). The ratio of LH to FSH activity is described in Figure 4c.

ANALOG MB192: (This changes the sequence DLRST of ANALOG MB140 to RDST).

Synthetic oligos 516 and 517 having the sequences:

5 516 5'-CGCGCTGTGTCGCGATAG-3' and  
517 3'-GACACAGCGCTATCATGA-5'

were ligated into pSVL-MB140\* by standard methods to give pSVL-MB192:

10

93 100  
C A L C R D S T T D C  
TGCGCGCTGTGTCGCGATAGTACTACCGACTGC  
ACGCGCGACACAGCGCTATCATGATGGCTGACG  
15 G^CGCGC TCGCGA A^GTACT  
BssHIII NruI ScaI

An NruI site was created and used for screening of the DNA construct during cloning. After its structure had been confirmed by DNA sequencing, pSVL-MB192 was co-transfected into COS-7 cells with pSVL-hCG-alpha. Media were concentrated and found to contain alpha, beta-heterodimer using the sandwich assay. This analog bound to LH and FSH receptors and stimulated cyclic AMP accumulation (Figures 5 and 7). The ratio of LH to FSH activity is described in Figure 4c.

ANALOG MB201: (This changes the sequence DLRST of ANALOG MB140 to RNST).

30

Synthetic oligos 518 and 519 having the sequences:

518 5'-CGCGCTGTGTCGGAATTC-3' and  
35 519 3'-GACACAGCCTTAAGATGA-5'

were ligated into pSVL-MB140\* by standard methods to give pSVL-MB201:



93 100  
 C A L C R N S T T D C  
 TGC GCGCTGTGTCGGAATTCTACTACCGACTGC  
 ACGCGCGACACAGCCTTAAGATGATGGCTGACG  
 5 G<sup>^</sup>CGCGC G<sup>^</sup>AATTC  
 BssHIII EcoRI

An EcoRI site was created and used for  
 screening of the DNA construct during cloning. After its  
 10 structure had been confirmed by DNA sequencing, pSVL-  
 MB201 was co-transfected into COS-7 cells with pSVL-hCG-  
 alpha. Media were concentrated and found to contain  
 alpha, beta-heterodimer using the sandwich assay. This  
 analog bound to LH and FSH receptors and stimulated  
 15 cyclic AMP accumulation (Figures 5 and 7). The ratio of  
 LH to FSH activity is described in Figure 4c.

ANALOG MB194: (This changes the sequence DLRST  
 of ANALOG MB140 to RSRT).  
 20

Synthetic oligos 520 and 521 having the  
 sequences:

520 5'-CGCGCTGTGTAGATCTCG-3' and  
 25 521 3'-GACACATCTAGAGCATGA-5'

were ligated into pSVL-MB140\* by standard methods to give  
 pSVL-MB194:

30 93 100  
 C A L C R S R T T D C  
 TGC GCGCTGTGTAGATCTCGTACTACCGACTGC  
 ACGCGCGACACATCTAGAGCATGATGGCTGACG  
 G<sup>^</sup>CGCGC A<sup>^</sup>GATCT  
 35 BssHIII BglIII

A BglIII site was created and used for screening  
 of the DNA construct during cloning. After its structure  
 had been confirmed by DNA sequencing, pSVL-MB194 was co-

5

10

15

15

20

25

30

ANALOG MB278: (This changes the sequence DLRST  
of ANALOG MB140 to RRRT).

Synthetic oligos 628 and 629 having the sequences:

628 5'-CGCGCTCTGTCGACGGCG-3' and  
5 629 3'-GAGACAGCTGCCGCATGA-5'

were ligated into pSVL-MB140\* by standard methods to give pSVL-MB278:

10 93 100  
C A L C R R R T T D C  
TGCGCGCTCTGTCGACGGCGTACTACCGACTGC  
ACGCGCGAGACAGCTGCCGCATGATGGCTGACG  
G^CGCGC G^TCGAC  
15 BssHII Sali

A Sali site was created and used for screening of the DNA construct during cloning. After its structure had been confirmed by DNA sequencing, pSVL-MB278 was co-  
20 transfected into COS-7 cells with pSVL-hCG-alpha. Media were concentrated and found to contain alpha, beta-heterodimer using the sandwich assay. This analog bound to LH and FSH receptors (Figures 4a and 4b). The ratio of LH to FSH activity is described in Figure 4c.

25

ANALOG MB279: (This changes the sequence DLRST of ANALOG MB140 to DDDT).

30 Synthetic oligos 630 and 631 having the sequences:

630 5'-CGCGTTATGCGATGACGA-3' and  
631 3'-AATACGCTACTGCTATGA-5'

35 were ligated into pSVL-MB140\* by standard methods to give pSVL-MB279:

35                   Oligos 365 and 510 were used in a PCR reaction using conditions similar to those described (29) with CF94-117 (27) as a primer. Oligos 508 and 368 were used in a second PCR reaction using pSVL-hCG-beta' as a primer. The products of the first and second reactions

were mixed and a third PCR reaction performed using oligos 363 and 364. The resulting DNA was then digested with XbaI and BamHI endonucleases, purified on agarose, and ligated into the XbaI/BamHI sites of pSVL. The sequence of the DNA between the XbaI and BamHI sites was confirmed by double stranded dideoxy sequencing. Note also that this introduced an SstII site which was used in further cloning.

```

10      93              100              110
      L C D S D S T D C T V R G L G P L T C D D P R F
      CTCTGCGACAGCGACAGTACTGACTGTACTGTCCGCGGCTTGGGTCCCTTGACCTGTGATGACCCCCGCTTC
      GAGACGCTGTCTGCTGTCATGACTGACATGACAGGCGCCGAACCCAGGGAACTGGACACTACTGGGGGCGAAG
      AGT^ACT   CCGC^GG
15      Scal      SstII

```

The portions of the molecule between residues 1-93 and 107-145 were identical in sequence to those of the hCG beta-subunit. This analog was co-transfected into COS-7 cells along with pSVL-hCG-alpha and the media were collected on the third day after transfection (27). The media were concentrated and analyzed by a sandwich immunoassay using antibodies A113 and radiolabeled B105. This analog combined with the alpha-subunit. It had relatively low ability to bind to either LH or FSH receptors (Table 6).

ANALOG PRM8: This is an analog identical to MB202 except that Thr97 of MB202 was replaced by Ser. PRM8 was made by replacing the XhoI/SstII fragment of MB202 with that of PRM3. It was made to learn if the higher than expected amount of LH activity found in MB202 relative to CF94-117 (Table 6) was due to the Thr at residue 97. This residue is Ser in hFSH beta-subunit. This analog was co-transfected into COS-7 cells along with pSVL-hCG-alpha and the media were collected for assay on the third day after transfection (27). The media were concentrated and analyzed by a sandwich immunoassay using antibodies A113 and radiolabeled B105.

5

15

30

**35**

100	110	120
C T V R G L G P S Y C S F G E F Q D S S S S K A		
TGCACTGTCCGGGTCTTGCGCCAAGCTATTGCAGCTTCGGCGAATTCAGGAAGTCTCTTCTCCTCAAAGGCC		
ACGTGACAGGCGCCAGAACCGGGTTCGATAACGTCGAAGCCGCTTAAGGTCTGAGGAGAAGGAGTTCCGG		
CCGCA <sup>+</sup> GG		G <sup>+</sup> AATTC
SstII		EcoRI

## ANALOG PRM2:

```

      100              110              120
      C T V R G L G P S F C S F G E F Q D S S S S K A
5    TGCACTGTCCGCGGTCTTGCCCAAGCTTTTGCAGCTTCGGCGAATTCAGGACTCCTCTTCTCAAAGGCC
      ACGTGACAGGCGCCAGAACCGGGTTCGAAACGTCGAAGCCGCTTAAGGTCTGAGGAGAAGGAGTTTCCGG
      CCGC^GG      A^AGCTT      G^AATTC
      SstII      HindIII      EcoRI

```

10 pSVL-PRM1 was co-transfected into COS-7 cells along with pSVL-hCG- $\alpha$  and the media were collected on the third day after transfection (27). The media were concentrated and analyzed by a sandwich immunoassay using antibodies A113 and radiolabeled B105. This analog  
15 combined with the  $\alpha$ -subunit. It had high ability to bind to FSH receptors and relatively low ability to bind to LH receptors (Table 6).

20 pSVL-PRM2 was co-transfected into COS-7 cells along with pSVL-hCG- $\alpha$  and the media were collected on the third day after transfection (27). The media were concentrated and analyzed by a sandwich immunoassay using antibodies A113 and radiolabeled B105. This analog  
25 combined with the  $\alpha$ -subunit. It had high ability to bind to FSH receptors and relatively low ability to bind to LH receptors (Table 6).

30 ANALOG PRM10: This is a full-length hCG  $\beta$ -subunit analog in which residues 101-106 are replaced with residues 95-100 of hFSH  $\beta$ -subunit. This analog was made by PCR using oligonucleotides 562 and 365 as PCR primers and MB135 as template. The resulting PCR product was cloned into the XhoI/SstII sites of PRM3. The  
35 structure of PRM10 was confirmed by double stranded dideoxy sequencing.

562: 5'-CCCAAGACCGCGGACAGTGCAGTCAGTGGTAGATCTGCG-3'

The sequence of PRM10 in the region 92-107 is:

```

          93              100              107
    L C R R S T T D C T V T G L G P
CTCTGCCGCAGATCTACCACTGACTGCACTGTCCGCGGTCTTGGGCCC
5  GAGACGGCGTCTAGATGGTGACTGACGTGACAGGCGCCAGAACCCGGG
      A^GATCT              CCGC^GG   GGGCC^C
      BglIII              SstII   ApaI

```

ANALOG MB197: This is an analog of CF101-109  
 10 with the deletion of Arg95. It was made by ligating the  
 large fragment obtained by digestion of pSVL-MB144 with  
 XbaI/BglIII with the small fragment obtained by digestion  
 of pSVL-MB194 using the same enzymes. The resulting  
 15 analog was only 113 residues in length and its sequence  
 between residues 88 and 99 is illustrated here.

```

          88          93              99
      C A L C R S T T D C
TGC GCGCTGTGTAGATCTACTACCGACTGC
20 ACGCGGACACATCTAGATGATGGCTGACG
      G^CGCGC   AGATCT
      BssHII   BglIII

```

pSVL-MB197 was expressed in COS-7 cells along  
 25 with pSVL-hCG-alpha. This analog had low ability to form  
 dimer with the alpha-subunit. The analog was readily  
 detected in a beta-subunit specific sandwich assay  
 employing antibodies B201 and radiolabeled B105 as  
 capture and detection antibodies, respectively (Table 5).  
 30 This indicated that it acquired the overall folding  
 pattern of the beta-subunit.

ANALOG MB198: This is an analog of CF101-109  
 with the insertion of a Gln at residue 94. It was made  
 35 by ligating the large fragment obtained by digestion of  
 MB194 with XbaI and BglIII to the small fragment obtained  
 by similar digestion of MB149 to create pSVL-MB194:



93 101  
 C A L C Q R S R T T D C  
 TGCGCGTTGTGTCAGAGATCTCGTACTACCGACTGC  
 ACGCGCAACACAGTCTCTAGAGCATGATGGCTGACG  
 5 AGATCT  
 BglIII

The ScaI and BssHII sites were destroyed and a  
 BglIII site was created. This information was used for  
 10 screening. After its structure had been confirmed by DNA  
 sequencing, pSVL-MB198 was co-transfected into COS-7  
 cells with pSVL-hCG-alpha. The analog was readily  
 detected in a beta-subunit specific sandwich assay  
 employing antibodies B201 and radiolabeled B105 as  
 15 capture and detection antibodies, respectively (Table 5).  
 This indicated that the beta-subunit folded.

ANALOG MB284: This is an analog of CF101-109  
 in which two residues have been deleted from the 93-100  
 20 region of the molecule. The analog was made by inserting  
 the following oligonucleotide cassette into the large  
 fragment remaining following digestion of pSVL-MB140  
 using BssHII and SstII.

25 632 5'-CGCGCTCTGTGCGACGGACCGACTGCACTGTCCGC-3' and  
 633 3'-GAGACAGCTGCCTGGCTGACGTGACAGG

This gave pSVL-MB284:

30 90 93 98 108 112  
 C A L C R R T D C T V R G L G P S Y C D D P R \*  
 TGCGCGCTCTGTGCGACGGACCGACTGCACTGTCCGCGTCTTGGGCCCTCTATTGTGATGATCCTAGATAA  
 ACGCGCGAGACAGCTGCCTGGCTGACGTGACAGGCGCCAGAACCCGGGAGAATAACACTACTAGGATCTATT  
 G^CGCGC G^TCGAC CCGC^GG GGGCC^C  
 35 BssHII SalI SstII ApaI

A SalI endonuclease restriction site was  
 created and the ScaI site was destroyed. This  
 information was used for screening. After its structure

had been confirmed by DNA sequencing, pSVL-MB284 was co-transfected into COS-7 cells with pSVL-hCG-alpha. Media were found to contain small amounts of alpha, beta-heterodimer using the A113/B105 sandwich assay. The  
5 analog was readily detected in a beta-subunit specific sandwich assay employing antibodies B201 and radiolabeled B105 as capture and detection antibodies, respectively (Table 5). This indicated that the beta-subunit folded.

10 ANALOG PRM26: The small fragment obtained by XhoI and SstII digestion of PRM10 was ligated to the large fragment obtained by XhoI and SstII digestion of PRM1 to give pSVL-PRM26. The resulting construct encoded residues 1-100 derived from the N-terminus of the hCG  
15 beta-subunit, residues 101-114 derived from the hFSH beta-subunit (i.e., corresponding to hFSH beta-subunit residues 95-108), and residues 115-145 derived from the C-terminus hCG beta-subunit. pSVL-PRM26 was co-transfected into COS-7 cells along with pSVL-hCG-alpha  
20 and the media were collected on the third day after transfection (27). The media were concentrated and analyzed by a sandwich immunoassay using antibodies A113 and radiolabeled B105. This analog combined with the alpha-subunit and the dimer had high LH activity  
25 (Table 6).

ANALOG MB261: (Changes residues CRNSTTDCTVR of ANALOG MB201 to CRRSTTDCGGR).

30 Synthetic oligos 599 and 600 having the sequences:

599 5'-CGCGCTGTGTAGAAAGATCTACTACCGACTGCGGCGGCCGC-3' and  
600 3'-GACACATCTTCTAGATGATGGCTGACGCCCGG-5'

35 were ligated into the large fragment obtained by BssHII and SstII digestion of pSVL-MB201 by standard methods to give pSVL-MB261:

```

          90          93          100
          C A L C R R S T T D C G G R
          TGCGCGCTGTGTAGAAGATCTACTACCGACTGCGGCGGGCCGC
          ACGCGCGACACATCTTCTAGATGATGGCTGACGCCGCCGGCG
5          G^CGCGC          A^GATCT
          BssHII          BglII

```

A BglII site was created and used for screening  
 of the DNA construct during cloning. After its structure  
 10 had been confirmed by DNA sequencing, pSVL-MB261 was co-  
 transfected into COS-7 cells with pSVL-hCG- $\alpha$ . Media  
 were concentrated and found to contain  $\alpha$ ,  $\beta$ -  
 heterodimer using the A113/B105 sandwich assay. This  
 analog bound LH receptors similar to hCG (Table 5).  
 15 Since 100ng did not inhibit the binding of  $^{125}$ I-hFSH to  
 human FSH receptors, it appears to have reduced activity  
 for FSH receptors.

ANALOG MB272: (Changes residues CGGRGLGPSYC of  
 20 ANALOG MB261 to CGGRKDGPSYC).

Synthetic oligos 624 and 625 having the  
 sequences:

```

25  624          5'-AAGGACGGGCC-3' and
    625          3'-CGTTCCTGC-5'

```

were ligated into the large fragment obtained by SstII  
 and ApaI digestion of pSVL-MB201 by standard methods to  
 30 give pSVL-MB272:

```

          90          93          100          110
          C A L C R R S T T D C G G R K D G P S Y C
          TGCGCGCTGTGTAGAAGATCTACTACCGACTGCGGCGGCCCAAGGACGGGCCCTCTATTGT
          ACGCGCGACACATCTTCTAGATGATGGCTGACGCCGCCGGCGTTCCTGCCCGGAGAATAACA
35          G^CGCGC          A^GATCT          G^GGCCC
          BssHII          BglII          ApaI

```

The SstII site found in pSVL-MB261 was destroyed and this was used for screening of the DNA construct during cloning. After its structure had been confirmed by DNA sequencing, pSVL-MB261 was co-transfected into COS-7 cells with pSVL-hCG-alpha. Media were concentrated and found to contain alpha, beta-heterodimer using the A113/B105 sandwich assay. This analog bound LH receptors similar to hCG (Table 5). Since 100ng did not inhibit the binding of <sup>125</sup>I-hFSH to human FSH receptors, it appears to have reduced activity for FSH receptors.

ANALOG MB275: (Changes residues CGGRGLGPSYC of ANALOG MB201 to CGGRKDGPSYC).

15

Oligos 624 and 625 were ligated into the large fragment obtained by SstII and ApaI digestion of pSVL-MB201 by standard methods to give pSVL-MB275:

20	90	93	100	110
	C	A	L	C
	R	N	S	T
	T	D	C	T
	V	R	K	D
	G	P	S	Y
	C			
	TGC GCG CTGTGT CGGAATTCTACTACCGACTGCACTGTCCGAAGGACGGGCCCTCTATTGT			
	ACGCGCGACACAGCCTTAAGATGATGGCTGACGTGACAGGCGTTCTGCCCGGAGAATAACA			
	G <sup>^</sup> CGCGC	G <sup>^</sup> AATTC	G <sup>^</sup> GGCCC	
25	BssHII	EcoRI	ApaI	

The SstII site found in pSVL-MB201 was destroyed and this was used for screening of the DNA construct during cloning. After its structure had been confirmed by DNA sequencing, pSVL-MB261 was co-transfected into COS-7 cells with pSVL-hCG-alpha. Media were concentrated and found to contain alpha, beta-heterodimer using the A113/B105 sandwich assay. This analog bound LH receptors similar to hCG (Table 5). Since 100ng did not inhibit the binding of <sup>125</sup>I-hFSH to human FSH receptors, it appears to have reduced activity for FSH receptors.

ANALOG MB283: (Changes residues CVTRGLGPSYC of CF101-109 to CVTRGLGPLT).

5 Synthetic oligos 626 and 627 having the sequences:

627: 5'-CTTAAGTTGTGATGATCCTAGATAAG-3' and  
626: 3'-CCGGGAATTGAACACTACTAGGATCTATTCCTAG-5'

10 were ligated with the large fragment of CF101-109 remaining following *Apa*I and *Bam*HI digestion to give pSVL-MB283:

100 110  
15 C T V R G L G P L T C D D P R \*  
TGCACCGTGAGAGGCCCTCGGCCCTTAAGTTGTGATGATCCTAGATAAGGATCC  
ACGTGGCACTCTCCGCACCCGGGAATTGAACACTACTAGGATCTATTCCTAGG  
AGG^CCT G^GATCC  
StuI BamHI

20

The *Sst*I site found in CF101-109 was destroyed and the loss of the site was used for screening of the DNA construct during cloning. After its structure had been confirmed by DNA sequencing, pSVL-MB261 was co-transfected into COS-7 cells with pSVL-hCG-*alpha*. Media were concentrated and found to contain *alpha*, *beta*-heterodimer using the A113/B105 sandwich assay. This analog bound LH receptors similar to hCG (Table 5). Since 100ng did not inhibit the binding of <sup>125</sup>I-hFSH to human FSH receptors, it appears to have reduced activity for FSH receptors.

30

#### ANALYSES

35 Quantification of analogs: The analogs were measured in sandwich immunoassays (28) using antibody A113 as a capture reagent and radiolabeled antibody B105 as a detection reagent. hCG was used as the standard. A113 binds to the *alpha*-subunit and B105 binds to the

beta-subunit. In some cases, the analogs were also quantified using a sandwich assay employing antibodies B109 or B107 as capture reagents and radiolabeled antibody B105 as a detection reagent. Antibodies B105, B107, and B109 bind to residues in the beta-subunit not changed in the analogs described here (30). For those analogs which had low activity in the A113/B105 sandwich assay, we measured the free beta-subunit to learn if it were being expressed. To do this we substituted antibody B201 for A113 and performed the remainder of the assay as described (28). B201 binds to an epitope on the beta-subunit much better than to hCG. To be certain that failure to obtain alpha, beta-heterodimers was not related to lack of alpha-subunit, we also measured the free alpha-subunit using antibodies A501 and radiolabeled A202 in place of A113 and radiolabeled B105. A501 binds free alpha-subunit much better than hCG.

FSH receptor binding: Binding of analogs to FSH receptors was measured using a CHO cell line that had been transfected with a cDNA encoding the human FSH receptor. This cell line was obtained from AAT, Randolph, MA and was grown in suspension culture in  $\alpha$ MEM containing 10% fetal bovine serum. On the day of the experiment cells were harvested, washed in phosphate buffered saline, and counted. Aliquots of 200,000 to 1,000,000 cells were incubated with  $^{125}\text{I}$ -hFSH and FSH standards or analogs to be tested for 1 hour at  $37\frac{1}{2}^{\circ}\text{C}$  in Krebs-Hepes buffer (28). The cells were collected by sedimentation at 1000xg for 10-15 min and the supernate containing the non-bound hormone was aspirated. The amount of radioactivity remaining in the cell pellets was determined in a gamma counter.

LH receptor binding: Binding of analogs to LH receptors was determined using a CHO cell line that had been transfected with a cDNA encoding the rat LH receptor (29) or with a cell line that had been transfected with the human LH receptor (31). The procedure used was

identical to that described for FSH receptor binding except that  $^{125}\text{I}$ -hCG and hCG were substituted for  $^{125}\text{I}$ -hFSH and hFSH.

5                   Cyclic AMP assays: CHO cells expressing rat LH receptors, human LH receptors, or human FSH receptors were incubated with analogs and hCG or hFSH for 10-15 minutes and then placed in a boiling water bath for 1 min. The cyclic AMP was measured using a cyclic AMP  
10 immunoassay (32).

#### ACTIVITIES OF ANALOGS

Interaction of analogs CF101-109 and CF94-117  
15 with human LH and FSH receptors (Figure 3): CF101-109 and CF94-117 are analogs of both hCG and hFSH. CF94-117 has been shown to bind FSH receptors (27) and here we illustrate that CF101-109 binds both LH and FSH  
20 receptors. Previous studies employed rat LH and FSH receptors and bovine FSH receptors (27). We expected that analogs of the human hormones which bound rat receptors would also bind to human receptors and initiate signal transduction. To confirm this prediction, we  
25 tested the abilities of CF94-117 (27) and CF101-109 to bind to human LH and FSH receptors stably expressed in Chinese hamster ovary CHO cells. Both CF94-117 and CF101-109 inhibited binding of  $^{125}\text{I}$ -hFSH to human FSH receptors (Figure 3). In addition, CF101-109 inhibited  
30 the binding of  $^{125}\text{I}$ -hCG to human LH receptors at about the same potency as hCG. CF101-109 and CF94-117 induced cyclic AMP accumulation in CHO cells expressing human FSH receptors, albeit with slightly lower potency than hFSH. It is not possible to provide exact potency estimates since the amounts of analogs used in these studies were  
35 determined against an hCG standard in sandwich immunoassays. As illustrated in Table 4, some antibody combinations give quantitatively different results than others. CF101-109 induced rounding of Y-1 cells transfected with genes that stably express LH receptors

(i.e., Y-1L cells) or FSH receptors (i.e., Y-1F cells). Rounding of Y-1 cells has long been correlated with responses to hormones such as ACTH for which the cells have ACTH receptors (33). Y-1F cells rounded in response to hFSH but not hCG whereas Y-1L cells rounded in response to hCG but not hFSH. This suggests that rounding is an index of response to any hormone which can induce cyclic AMP formation in cells derived from the original Y-1 line. Since both Y-1F and Y-1L cells respond to CF101-109, this analog must be functionally interacting with FSH and LH receptors in these cells. In addition, the Y-1 cells transfected with FSH receptors made progesterone in response to CF101-109 and hFSH but not to hCG (Figure 4). This further suggests that CF101-109 can elicit a physiological response such as steroid production.

Abilities of analogs of CF101-109 to combine with alpha-subunit. To determine if analogs of CF101-109 would combine with the alpha-subunit to form a heterodimer, we subjected culture media from transfected cells to sandwich assays that detected alpha, beta heterodimers. All results were quantified using an hCG standard. Most assays employed A113 (i.e., an antibody specific for the alpha-subunit) and B105 (i.e., an antibody specific for the beta-subunit). Although B105 does not bind to hFSH, it has been shown to bind to CF94-117 (27). This is an analog in which all residues between 94 and 117 are derived from hFSH beta-subunit. Therefore, B105 binds a part of the beta-subunit outside the region that was modified in the studies described here. In some studies, we also employed antibodies B107 or B109 and B105. B107 and B109 have also been shown to bind to portions of the -subunit outside the region modified in these studies (30). Although the antibodies used in these assays were chosen on the basis of the fact that they recognized different regions of the hormone than was modified in these studies, slight changes in the conformations of the alpha- and/or beta-subunits caused



by the mutations may have influenced binding of the analogs to the antibodies (B107 and B110 which are known to be sensitive to conformation of the *beta*-subunit which occurs during formation of the *alpha*, *beta*-heterodimer (30)). We observed differences in the absolute amounts of analogs depending on the sandwich immunoassays which were employed. As a rule, sandwich assays using B109 and B105 gave lower estimates than those assays which employed other antibody combinations (Table 4). None the less, differences in the amounts of material present in the media were not correlated with the activities of the analogs in either LH receptor or FSH receptor assays. It should also be noted that the same batches of analogs were used in FSH and LH receptor assays. Thus, estimates in the amounts of analog present in culture medium do not enter into the calculation of the ratio of LH to FSH activity. Except as noted in Table 4, the data illustrated in all tables and figures were obtained using the A113/B105\* sandwich assay.

20

The results of sandwich immunoassays indicated that all the analogs we made except for MB140, MB197, MB198, and MB284 formed *alpha*, *beta* dimers when co-expressed with the human *alpha*-subunit. Cells expressing the human *alpha*-subunit and MB140, MB197, MB198, or MB284 *beta*-subunits did not produce much *alpha*, *beta*-heterodimer. These were the only analogs in this series which had a different number of residues in the region between Cys93 and Cys100 than is found in all naturally occurring hormone *beta*-subunits (34). Every analog we prepared which contained 6 amino acid residues between Cys93 and Cys100 combined with the *alpha*-subunit and was detected in A113/B105\* sandwich immunoassays. These included analogs which contain a glycosylation signal at residue 94 (i.e., MB145), residue 95 (i.e., MB201), or residue 96 (i.e., MB148) as well as analogs with three positive charges (i.e., MB278) or four negative charges (i.e., MB279) between Cys93 and Cys100. Thus, we anticipate that mutations in this region will be

EFFECTS OF MUTATIONS IN RESIDUES 94-97 OF CF101-109 ON LH  
AND FSH ACTIVITY

As illustrated in Figure 3, CF101-109 binds to human LH receptors and human FSH receptors. In the studies reported here we have used the rat LH receptor and human LH and FSH receptors to test the activities of the CF101-109 analogs. Modification of residues in the region of the molecule between 94-97 altered the affinities of CF101-109 analogs for LH receptors more than it altered their affinities for FSH receptors (Figure 3). The charge of the amino acids in this region was correlated with LH activity (Figures 4a and 4c). Binding to LH receptors was enhanced when at least one positive charge or no negative charges were present in the 94-97 region. Thus, while analogs MB154 (ASDT), MB152 (DSAT), MB202 (DSDT), and MB279 (DDDT) competed well with  $^{125}\text{I}$ -hFSH for binding to FSH receptors, they had the lowest ability to compete with  $^{125}\text{I}$ -hCG for binding to LH receptors. Inclusion of a positive charge or replacement of a negative charge with one positive charge resulted in much better binding to LH receptors without substantially altering binding to FSH receptors. Thus, analogs MB151 (DSRT) and MB147 (RSRT) were more active than MB154 (ASDT), MB152 (DSAT), MB202 (DSDT) or MB279 (DDDT) in the LH receptor assay. Elimination of all negative charges was sufficient to increase the LH activity. Thus MB155 (QIST) was more active than MB154 (ASDT), MB152 (DSAT), MB202 (DSDT) or MB279 (DDDT). As a rule, analogs with no negative charges had the greatest ability to bind to LH receptors. These included MB149 (QRST), MB150 (RQST), MB145 (NRST), MB201 (RNST) and MB194 (RSRT). With some exceptions, notably the lower activity of MB147 (RSRT)

relative to that of MB151 (DSRT), the locations of the charged residues had only a small influence on binding to LH receptors. For example, both MB149 (QRST) and MB150 (RQST) were very active even though they have Arg residues at different positions. Likewise, the ratio of LH/FSH activity of MB151 (DSRT) was only slightly less than that of MB147 (RSDT). While most position effects were relatively minor, the residue in which a negative charge had the greatest influence on LH receptor binding appeared to be number 96. These results suggest that analogs having high FSH specificity can be prepared by substituting negative charges in the region of the molecule between Cys93 and Cys100. Use of positive charges in this region is expected to lead to enhanced LH receptor interactions.

Results of FSH Binding Assays (Figures 3 and 4b). In general, substitutions in the region between 94 and 97 had a much lower effect in the FSH binding assays than in the LH binding assays. Most analogs were more than 20% as active as FSH in this assay. The major exception to this statement was analog MB153 (DRDT). This analog was more active in LH receptor binding assays than in FSH receptor binding assays even though it had negative charges at residues 94 and 96.

Results of LH-receptor induced cyclic AMP formation assays (Figure 7). Most analogs which bound to LH receptors were capable of stimulating cyclic AMP formation. Their potency relative to hCG was similar to their potency relative to hCG in receptor binding assays.

Results of FSH-receptor induced cyclic AMP formation assays (Figure 7). Most analogs which bound to FSH receptors were capable of stimulating cyclic AMP formation. Their potency in this assay was only approximately 10% of that of their expected potency based on their abilities to bind to FSH receptors. CF94-117 was also somewhat less capable of eliciting cyclic AMP

accumulation in CHO cells bearing FSH receptors. This observation is surprising because CF94-117 was nearly equally effective as FSH in inducing steroidogenesis in cultured granulosa cells (27), a response presumed to be mediated by cyclic AMP (35). This, in the case of FSH receptors, we anticipate that the ability of all analogs to stimulate granulosa cell steroidogenesis will be better than their abilities to stimulate cyclic AMP accumulation in cells that stably express the FSH receptor.

#### EFFECTS OF MUTATIONS IN RESIDUES 101-114 OF CF101-109 ON LH AND FSH ACTIVITY

The region of the *beta*-subunit between residues 108 and the C-terminus was found to influence the specificity of hormone binding. We observed that analog MB202 had at least 10-fold greater ability than CF94-117 to bind to LH receptors (Table 6). The differences between MB202 and CF94-117 include the substitution of Thr for Ser97 and the sequence AspAspProArg for SerPheGlyGluMetLysGlu at the C-terminus. Previous studies indicated that CF94-114 had equal activity as CF94-117 (27) suggesting that the three residues at the very C-terminus of hFSH (i.e., MetLysGlu) did not have a role in binding specificity. To identify a role for Ser97 in binding specificity, we prepared PRM8. This analog differed from MB202 only in the presence of Ser at position 97. PRM8 bound to LH receptors better than CF94-117 suggesting that Ser97 was not important for discriminating LH and FSH receptors. It also suggested that residues between 111 and 114 or 111 and 117 had an important role in suppressing binding of FSH to LH receptors. To examine an influence of the C-terminus, we prepared PRM1 and PRM2. PRM1 is similar in structure to CF94-114 (27) except that it also contains residues 115-145 of the hCG C-terminus. PRM1 had similar abilities as CF94-117 to distinguish LH and FSH receptors. This suggested that FSH residues 111, 112, 114, and/or 114

were needed to reduce LH receptor binding to the level observed in CF94-114 or CF94-117. The influence of residues 111-114 on LH receptor interaction is lower than that of residues between 94-97. By comparing the activities of PRM26 (Table 6), PRM1 (Table 6), and CF94-97, 108-114 (Table 3), it can be seen that substituting hFSH beta-subunit sequences 105-108 for residues 111-114 does not appear to substantially alter the ability of the analog to bind to LH receptors when residues 94-97 or residues 101-106 of the analogs are derived from hCG beta-subunit. However, CF94-114, CF94-117, PRM1, and PRM2 have low LH activity. This shows that substitution of hFSH beta-subunit residues 105-108 for analogs 111-114 can modulate LH receptor activity when residues 94-108 are derived from hFSH beta-subunit. The ability of PRM2 to bind to FSH receptors suggests that substitution of Phe for Tyr109 does not alter binding specificity or affinity.

In further support of a role of residues between 101-109 in FSH receptor binding, we observed that PRM3, an analog which differed from PRM1 by the presence of hCG beta-subunit sequences in the region between residues 108-114, bound poorly to LH receptors yet had unexpectedly low affinity for FSH receptors. The low binding of PRM3 to LH receptors was caused by the presence of negative charges in the region of the molecule between residues 94 and 97. Since CF101-109 binds FSH receptors well and PRM3 has low affinity for FSH receptors, it appears that residues 108 and 109 contribute to the ability of the heterodimer to recognize LH and FSH receptors. Suppression of LH binding activity in PRM8 and MB202 requires modification of amino acids between residues 111 and 114. The ability of PRM2 to bind to FSH receptors suggests that substitution of Phe for Tyr109 does not alter binding specificity or affinity.

The following are the preferred analogs of the invention:

5 CF101-109, MB278: These two analogs give high binding to both LH and FSH receptors and are active in inducing a biological response.

PRM1, PRM2: These two analogs have the greatest selectivity to FSH receptors.

10

MB202: This analog has high FSH activity with small amounts of LH activity.

15 This invention teaches the regions of the glycoprotein hormone *beta*-subunit which controls the LH and FSH receptor binding activity. These regions are found between the 10th and 12th cysteine residues. A secondary determinant is also found within a few residues C-terminal of the 12th cysteine. Since the overall  
20 structure of the glycoprotein hormones has been conserved during evolution, it is expected that the same regions of all vertebrate gonadotropins are involved in receptor binding specificity. Thus, to obtain analogs with high LH and FSH activity, one would prepare *alpha*, *beta*-heterodimers from the glycoprotein *alpha*-subunit and a  
25 *beta*-subunit analog of LH in which the region between the 11th and 12th cysteines was substituted by the residues found between the 11th and 12th cysteines of FSH. To reduce the LH activity of this analog, one would  
30 substitute residues between the 10th and 11th cysteines with those found between the 10th and 11th cysteines of FSH. In the case of mammalian hormones, one can also reduce the positive charges and/or increase the negative charges in this region. To reduce the LH activity  
35 further in this analog, one would substitute the residues immediately C-terminal of the 12th cysteine with those of FSH. To influence the FSH activity of this analog, one would alter the residues between the 11th and 12th cysteine. One effective strategy in reducing FSH

activity is to employ residues from LH. By manipulating the residues between the 10th and 12th cysteines in the LH or CG beta-subunits in this way; one can prepare analogs of glycoprotein hormones with nearly any desired receptor binding specificity. In addition, by use of positive charges between the 10th and 11th cysteines, it is possible in mammalian hormone to enhance the binding of the analog to either LH or FSH receptors. Since residues between the 1st and 10th cysteine residues of the beta-subunit do not have a great influence on receptor binding specificity, the composition of residues in this region will be of much less importance.

TABLE 4\*

15

## Antibody Combination

	Analog	B109/B105*	B107/B105*	A113/B105*
		(ug/ul)	(ug/ul)	(ug/ul)
20	MB144 (DRST)	0.17	1.01	0.41
	MB145 (NRST)	0.40	1.53	1.79
	MB146 (RSST)	0.12	0.15	0.11
	MB147 (RSDT)	0.02	0.03	0.04
	MB148 (RSNT)	0.00	0.01	0.03
25	MB149 (QRST)	0.16	0.87	1.45
	MB151 (DSRT)	0.09	1.67	2.33
	MB152 (DSAT)	0.03	1.72	0.45
	MB153 (DRDT)	0.00	0.12	0.10
	MB154 (ASDT)	0.06	1.88	3.08
30	MB155 (QIST)	0.10	1.32	1.77
	MB140 (DLRST)	0.00	0.00	0.00

\* Comparison of estimates of the amounts of analog present in unconcentrated culture medium (ug/ul) by sandwich assays employing antibody A113-B105 and B109-B105 combinations and hCG as a reference standard. Not all analogs were examined in the B109/B105\* or B107/B105\* sandwich assays. We used the values obtained from the A113/B105\* assay as the basis for all subsequent studies.

5



TABLE 5 \*

Analog	Unconcentrated		Unconcentrated		Unconcentrated		50-Fold Concentrated	
	alpha-subunit (ng/ml) (A501/A202 Assay)	beta-subunit (ng/ml) (B201/A105 Assay)	alpha-subunit (ng/ml) (A113/B105 Assay)	beta-subunit (ng/ml) (A113/B105 Assay)	alpha, beta-heterodimer (ng/ml) (A113/B105 Assay)	alpha, beta-heterodimer (ng/ml) (A113/B105 Assay)	alpha, beta-heterodimer (ng/ml) (A113/B105 Assay)	alpha, beta-heterodimer (ng/ml) (A113/B105 Assay)
MB140	4.8 +/- 0.9	18.5 +/- 2.2	3.2 +/- 0.6	58.6 +/- 3.2				
MB197	4.1 +/- 0.3	14.7 +/- 2.8	-2.1 +/- 1.3	6.2 +/- 1.8				
MB198	3.7 +/- 0.6	12.9 +/- 4.6	-1.6 +/- 1.3	7.7 +/- 3.2				
MB284	7.9 +/- 2.8	32.0 +/- 10.3	-2.4 +/- 1.1	5.0 +/- 2.6				
CF101-109	5.7 +/- 0.7	21.8 +/- 3.0	9.6 +/- 2.3	offscale				

\* Influence of the size of the beta-subunit region between Cys93 and Cys100 on the ability of analogs to form alpha, beta heterodimers when they are co-expressed in COS-7 cells with pSVL-hCG-alpha, an expression vector encoding the human alpha-subunit.

TABLE 6 \*

	Analog	LH Binding Activity (relative to hCG)	FSH Binding Activity (relative to hFSH)
5			
	CF94-117	0.001	0.5
	CF94-114*	0.001	1
	PRM1	<0.001	0.1
	PRM2	<0.001	0.2
10	PRM8	0.08	not measured
	MB202	0.06	0.4
	PRM3	0.02	<0.001
	PRM26	1	not measured

15 \* Effect of residues near Cys110 on receptor binding specificity. The analogs were tested for their abilities to bind to LH and FSH receptors.

20 Notes: The values illustrated were determined by measuring the ability of the analogs to inhibit the binding of  $^{125}\text{I}$ -hCG to intact cells stably expressing LH receptors and the binding of  $^{125}\text{I}$ -hFSH to intact cells stably expressing FSH receptors. \*The values for CF94-  
 25 114 were taken from our earlier publication and were determined using tissue homogenates (27).

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20                   The invention being thus described, it will be  
obvious that the same may be varied in many ways. Such  
variations are not to be regarded as a departure from the  
spirit and scope of the invention and all such  
modifications are intended to be included within the  
25                   scope of the following claims.

W claim:

1. An alpha, beta-heterodimeric polypeptide having binding affinity to vertebrate luteinizing hormone (LH) receptors and vertebrate follicle stimulating hormone (FSH) receptors comprising a glycoprotein hormone alpha-subunit polypeptide and a non-naturally occurring beta-subunit polypeptide, wherein the beta-subunit polypeptide is a chain of amino acids comprising the following four joined subsequences:

(a) a first subsequence homologous to the amino acid sequence of residues 1-93 of the beta-subunit selected from the group consisting of human chorionic gonadotrophin (hCG), vertebrate luteinizing hormone (LH), vertebrate follicle stimulating hormone (FSH), and vertebrate thyroid stimulating hormone (TSH);

(b) a second subsequence homologous to the amino acid sequence of residues 94-97 of the beta-subunit selected from the group consisting of human chorionic gonadotrophin (hCG) and vertebrate luteinizing hormone (LH);

(c) a third subsequence homologous to the amino acid sequence of residues 98-100 of the beta-subunit selected from the group consisting of human chorionic gonadotrophin (hCG), vertebrate luteinizing hormone (LH), vertebrate follicle stimulating hormone (FSH), and vertebrate thyroid stimulating hormone (TSH); and

(d) a fourth subsequence homologous to the amino acid sequence of residues 101-110 of the beta-subunit of vertebrate follicle stimulating hormone.

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2. The alpha, beta-heterodimeric polypeptide according to claim 1, wherein the glycoprotein hormone alpha-subunit polypeptide is a human alpha-subunit polypeptide.

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3. The alpha, beta-heterodimeric polypeptide according to claim 1, wherein the glycoprotein hormone alpha-subunit polypeptide is the alpha-subunit of hCG.

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4. The alpha, beta-heterodimeric polypeptide according to claim 1, wherein the first subsequence is homologous to residues 1-93 of the beta-subunit of human chorionic gonadotrophin (hCG), the second subsequence is homologous to residues 94-97 of the beta-subunit of human

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chorionic gonadotrophin (hCG), the third subsequence is homologous to residues 98-100 of the beta-subunit of human chorionic gonadotrophin (hCG), and the fourth subsequence is homologous to residues 101-110 of the beta-subunit of vertebrate follicle stimulating hormone.

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5. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and the alpha, beta-heterodimeric polypeptide according to claim 1.

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6. An alpha, beta-heterodimeric polypeptide having greater binding affinity for vertebrate follicle stimulating hormone (FSH) receptors than for vertebrate luteinizing hormone (LH) receptors comprising a glycoprotein hormone alpha-subunit polypeptide and a non-naturally occurring beta-subunit polypeptide, wherein the

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beta-subunit polypeptide is a chain of amino acids comprising the following four joined subsequences:

(a) a first subsequence homologous to the amino acid sequence of residues 1-93 of the beta-subunit selected from the group consisting of human chorionic gonadotrophin (hCG), vertebrate luteinizing hormone (LH), vertebrate follicle stimulating hormone (FSH), and vertebrate thyroid stimulating hormone (TSH);

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(b) a second subsequence comprising 4 amino acids for residues 94-97;

5 (c) a third subsequence comprising 3 amino acids for residues 98-100; and

(d) a fourth subsequence homologous to the amino acid sequence of residues 101-110 of the beta-  
10 subunit of vertebrate follicle stimulating hormone.

7. An alpha, beta-heterodimeric polypeptide having binding affinity to follicle stimulating hormone (FSH) receptors and luteinizing hormone (LH) receptors  
15 comprising a glycoprotein hormone alpha-subunit polypeptide and a non-naturally occurring beta-subunit polypeptide, wherein the beta-subunit polypeptide is a chimera comprised of amino acids 1-100 of any vertebrate glycoprotein hormone homologous to amino acids found in  
20 residues 1-100 of human chorionic gonadotropin and any 1-20 amino acids which binds LH receptors better than FSH receptors and has biological activity.

## AMENDED CLAIMS

[received by the International Bureau on 30 November 1992 (30.11.92);  
original claims 1-7 replaced by amended claims 1-8 (3 pages)]

1. An *alpha*, *beta*-heterodimeric polypeptide having binding affinity to vertebrate luteinizing hormone (LH) receptors and vertebrate follicle stimulating hormone (FSH) receptors consisting essentially of a glycoprotein hormone *alpha*-subunit polypeptide and a non-naturally occurring *beta*-subunit polypeptide, wherein the *beta*-subunit polypeptide is a chain of amino acids comprising the following four joined subsequences:

(a) a first subsequence homologous to the amino acid sequence of residues 1-93 (hCG numbering) of the *beta*-subunit selected from the group consisting of human chorionic gonadotrophin (hCG), vertebrate luteinizing hormone (LH), vertebrate follicle stimulating hormone (FSH), and vertebrate thyroid stimulating hormone (TSH);

(b) a second subsequence homologous to the amino acid sequence of residues 94-97 (hCG numbering) of the *beta*-subunit selected from the group consisting of human chorionic gonadotrophin (hCG) and vertebrate luteinizing hormone (LH);

(c) a third subsequence homologous to the amino acid sequence of residues 98-100 (hCG numbering) of the *beta*-subunit selected from the group consisting of human chorionic gonadotrophin (hCG), vertebrate luteinizing hormone (LH), vertebrate follicle stimulating hormone (FSH), and vertebrate thyroid stimulating hormone (TSH); and

(d) a fourth subsequence homologous to the amino acid sequence of residues 95-104 (FSH numbering) of the *beta*-subunit of vertebrate follicle stimulating hormone.

2. The *alpha*, *beta*-heterodimeric polypeptide according to claim 1, wherein the glycoprotein hormone *alpha*-subunit polypeptide is a human *alpha*-subunit polypeptide.

3. The *alpha*, *beta*-heterodimeric polypeptide according to claim 1, wherein the glycoprotein hormone *alpha*-subunit polypeptide is the *alpha*-subunit of hCG.

4. The *alpha, beta*-heterodimeric polypeptide according to claim 1, wherein the first subsequence is homologous to residues 1-93 of the *beta*-subunit of human chorionic gonadotrophin (hCG), the second subsequence is homologous to residues 94-97 of the *beta*-subunit of human chorionic gonadotrophin (hCG), the third subsequence is homologous to residues 98-100 of the *beta*-subunit of human chorionic gonadotrophin (hCG), and the fourth subsequence is homologous to residues 101-110 of the *beta*-subunit of vertebrate follicle stimulating hormone.

5. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and the *alpha, beta*-heterodimeric polypeptide according to claim 1.

6. An *alpha, beta*-heterodimeric polypeptide having greater binding affinity than luteinizing hormone (LH) for vertebrate follicle stimulating hormone (FSH) receptors consisting essentially of a glycoprotein hormone *alpha*-subunit polypeptide and a non-naturally occurring *beta*-subunit polypeptide, wherein the *beta*-subunit polypeptide is a chain of amino acids comprising the following four joined subsequences:

(a) a first subsequence homologous to the amino acid sequence of residues 1-93 (hCG numbering) of the *beta*-subunit selected from the group consisting of human chorionic gonadotrophin (hCG), vertebrate luteinizing hormone (LH), vertebrate follicle stimulating hormone (FSH), and vertebrate thyroid stimulating hormone (TSH);

(b) a second subsequence comprising 4 amino acids for residues 94-97 (hCG numbering);

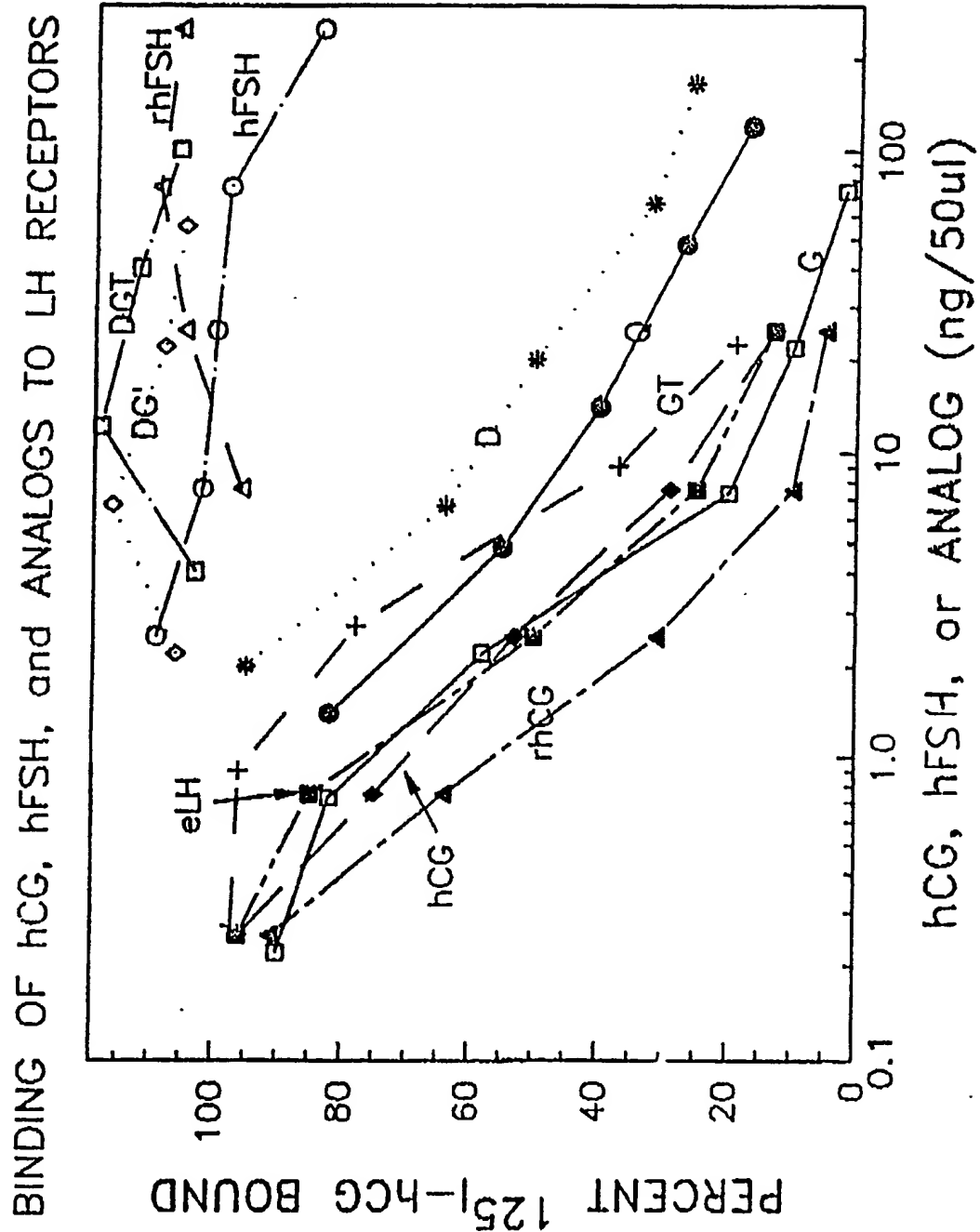
(c) a third subsequence comprising 3 amino acids for residues 98-100 (hCG numbering); and

(d) a fourth subsequence homologous to the amino acid sequence of residues 95-104 (FSH numbering) of the *beta*-subunit of vertebrate follicle stimulating hormone.

7. The *alpha*, *beta*-heterodimeric polypeptide according to claim 6, wherein residues 94-97 are other than Asp-Ser-Asp-Ser.

8. An *alpha*, *beta*-heterodimeric polypeptide having greater binding affinity than luteinizing hormone for follicle stimulating hormone (FSH) receptors consisting essentially of a glycoprotein hormone *alpha*-subunit polypeptide and a non-naturally occurring *beta*-subunit polypeptide, wherein the *beta*-subunit polypeptide is a chimera comprised of amino acids 1-100 of any vertebrate glycoprotein hormone homologous to amino acids found in residues 1-100 of human chorionic gonadotropin and any 1-20 amino acids which binds LH receptors better than FSH receptors and has biological activity.

FIGURE 1



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FIGURE 2

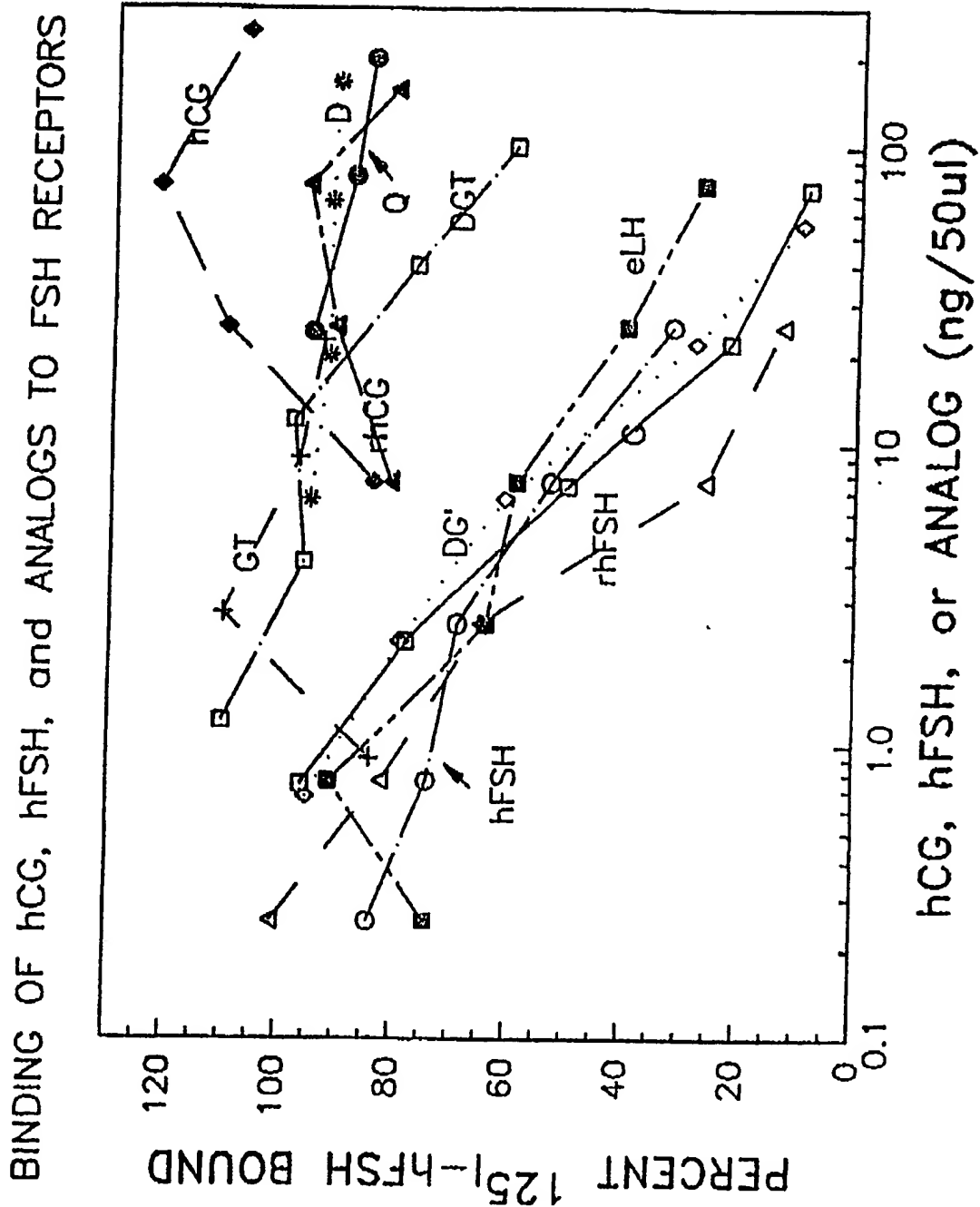
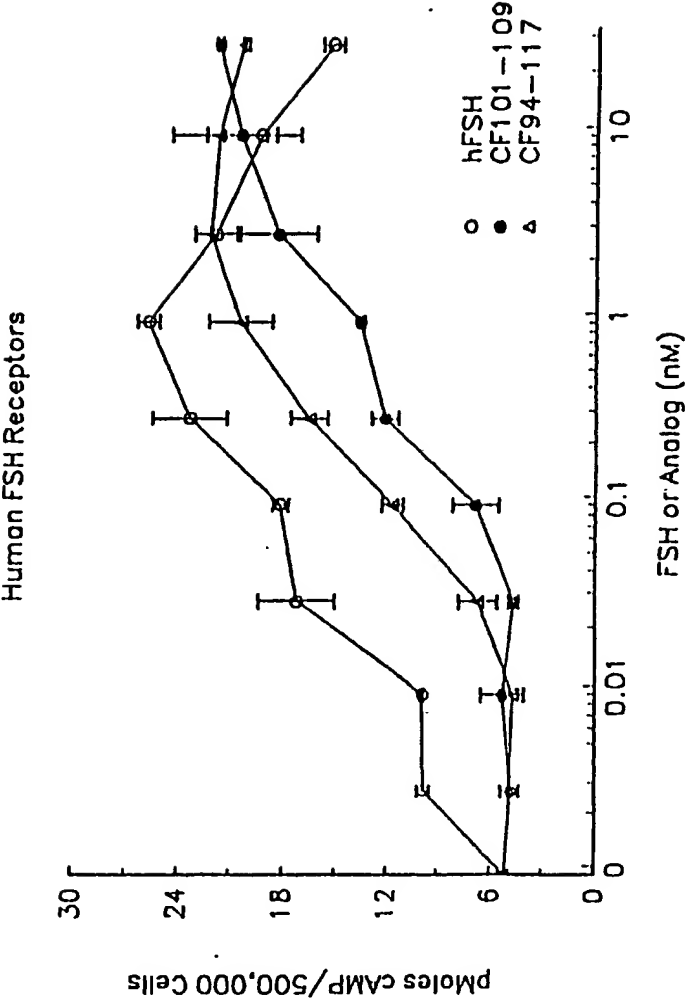
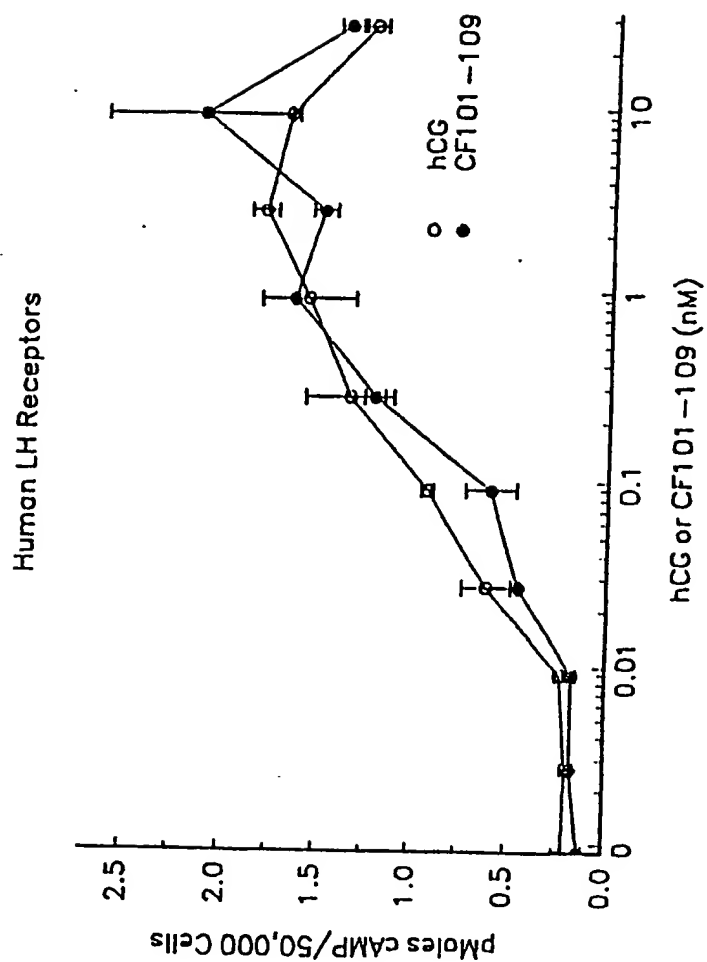


FIGURE 3A



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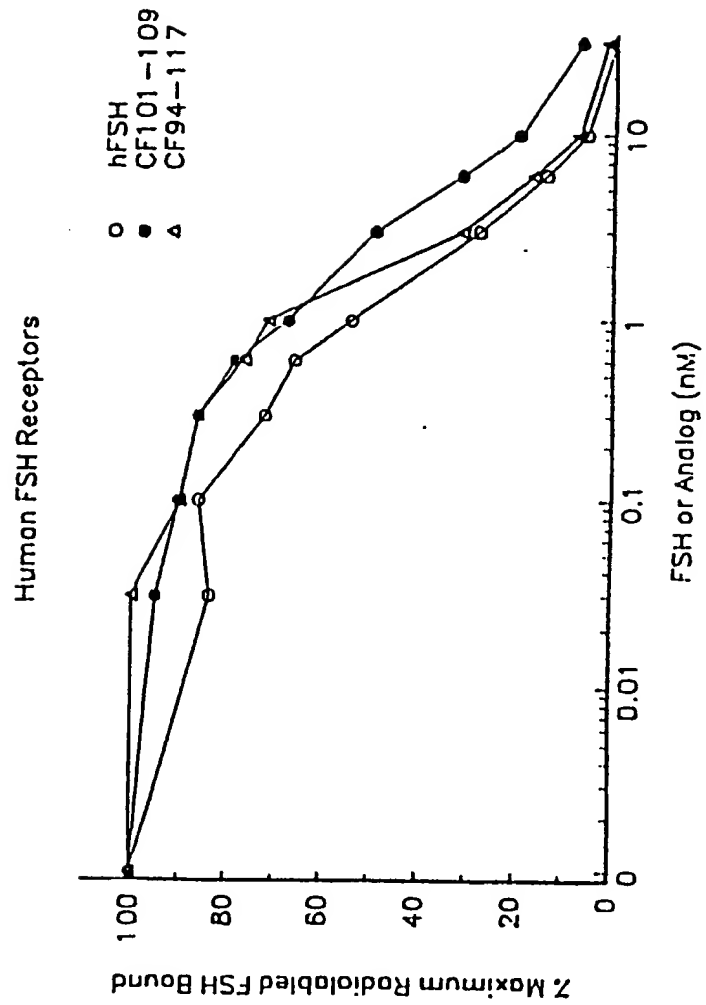
FIGURE 3B





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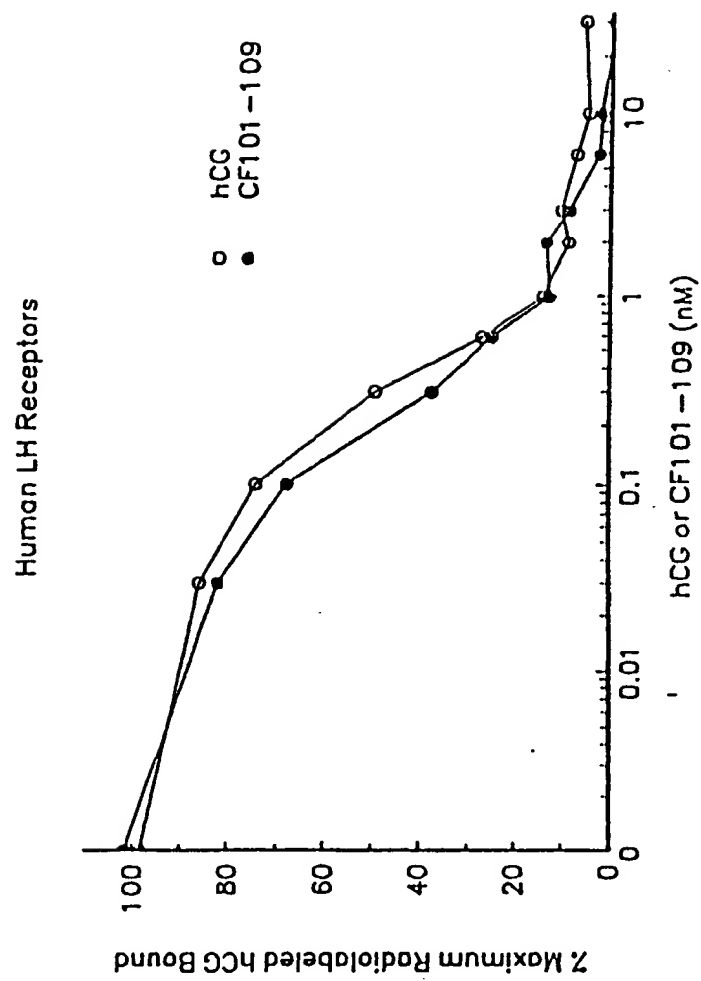
FIGURE 3C



SUBSTITUTE SHEET

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FIGURE 3D



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FIGURE 4

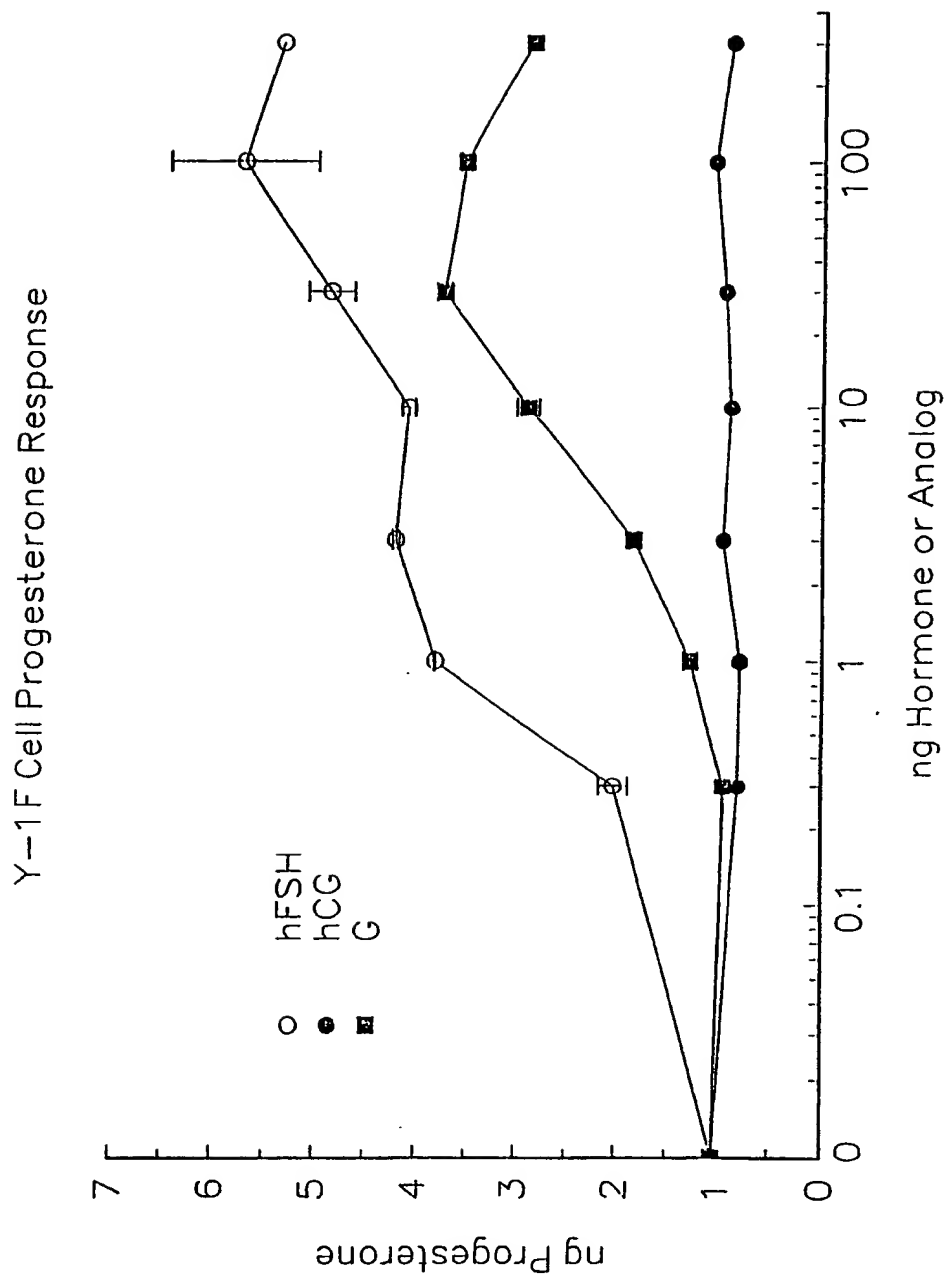


FIGURE 5

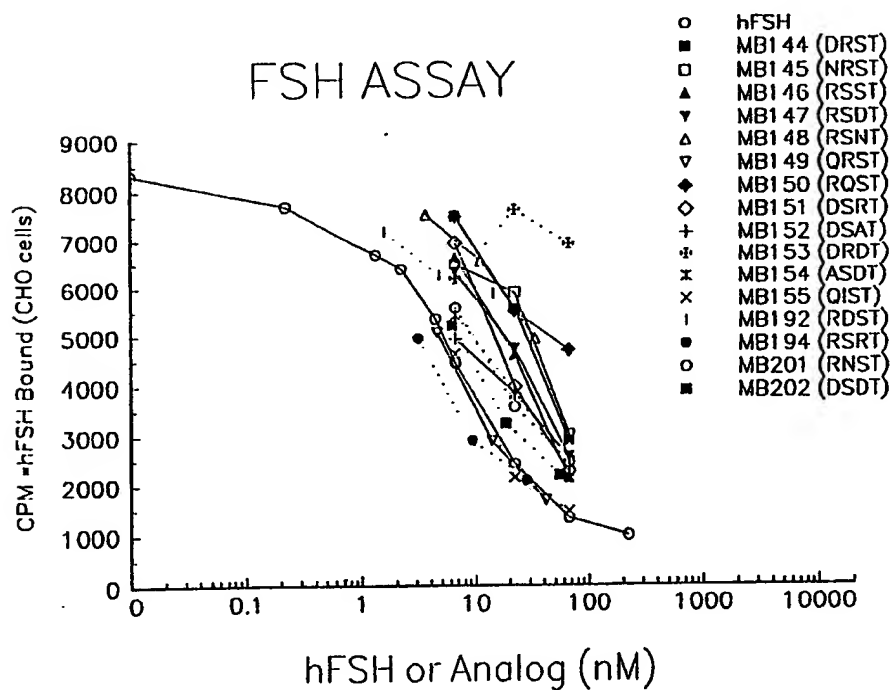
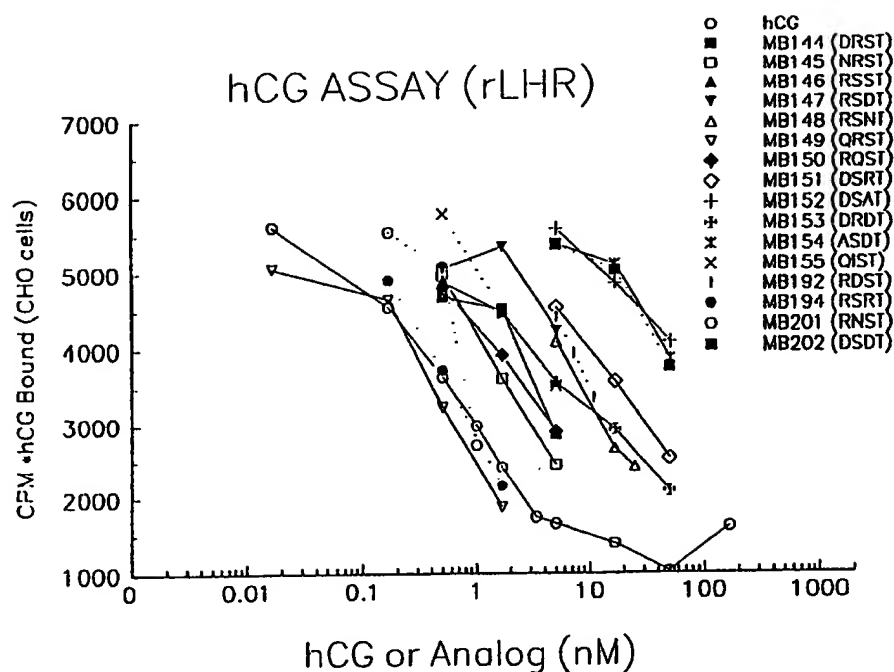


FIGURE 6A

Effect of Charge on FSH Receptor Binding

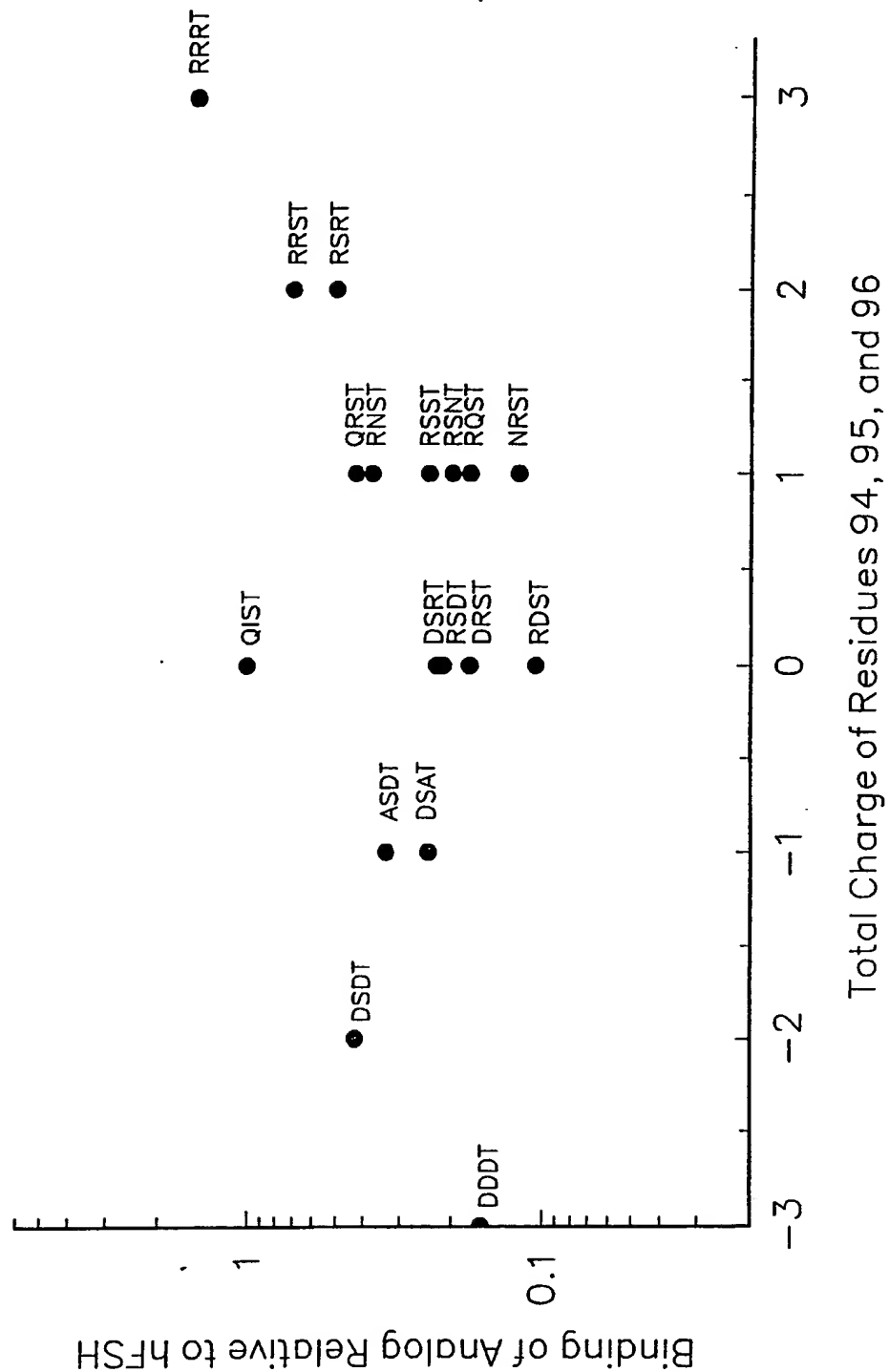


FIGURE 6B

# Effect of Charge on LH Receptor Binding

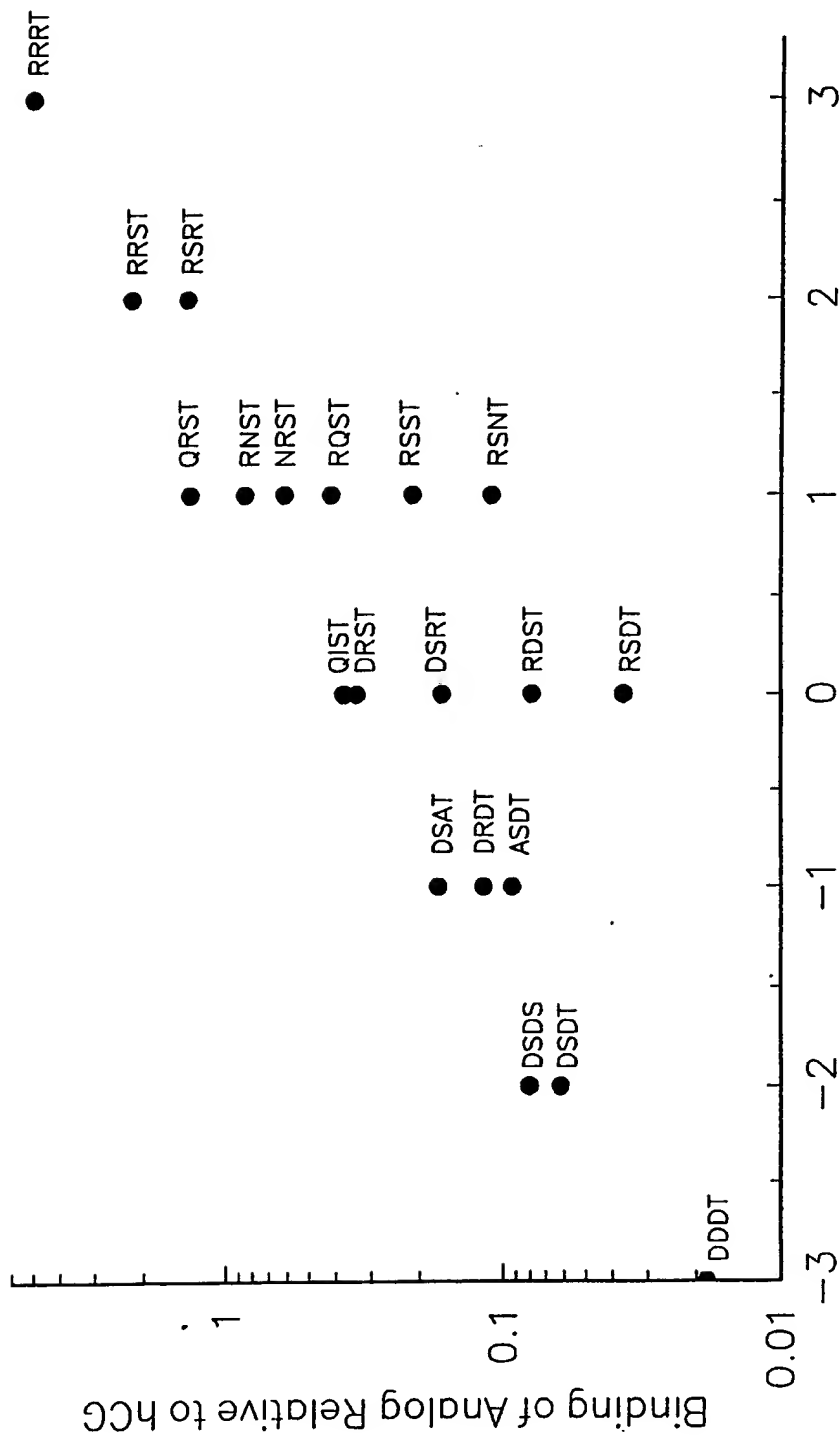


FIGURE 6C

Effect of Charge on Ratio of LH to FSH Activity

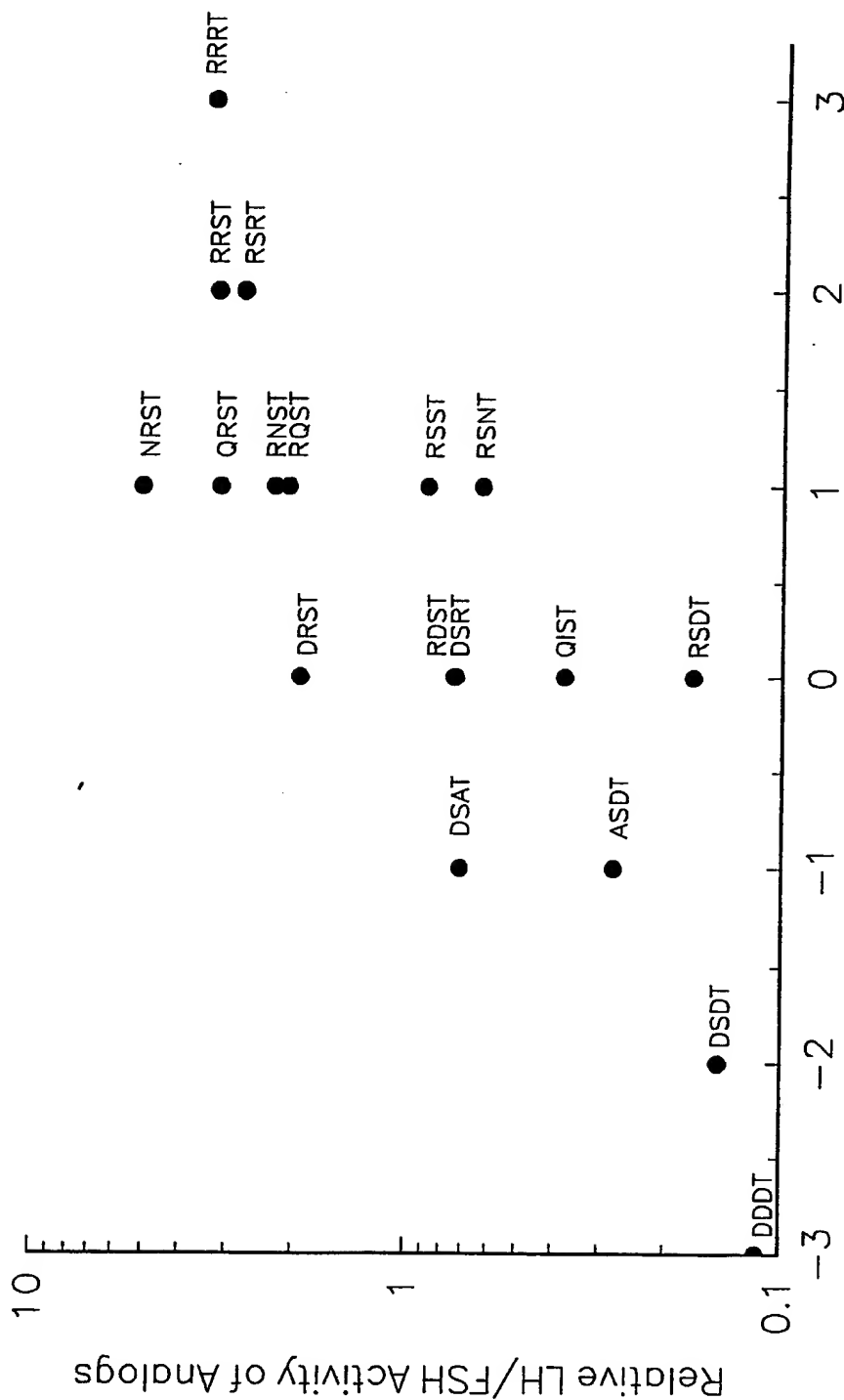
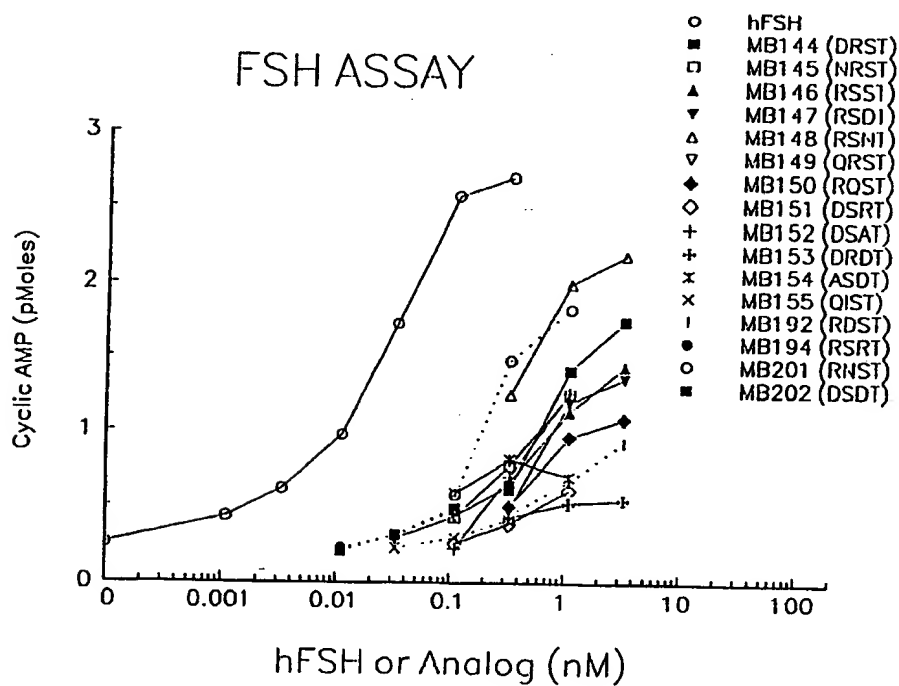
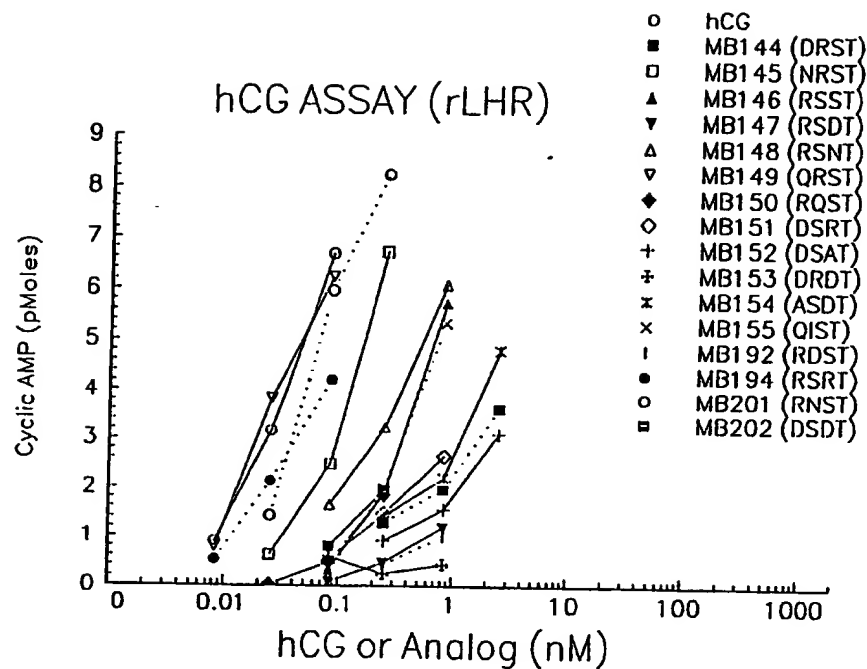


FIGURE 7





## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/05207**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :C07K 3/00, 13/00; A61K 37/24, 37/38

US CL :530/330,395,397,398,399,854; 514/8,12

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350,395,397,398,399,854; 514/8,12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS AND SEQUENCE SEARCH

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ENDOCRINE REVIEW, VOLUME 12, No. 1, Issued 1991, B.D. Murphy et al., "Equine Chorionic Gondotropin", pages 27-44.	1-7
A	Proceedings of the National Academy of Sciences, Volume 88, issued February 1991, R.K. Campbell et al., "Conversion of Human Choriogonadotropin into a follitropin by Protein Engineering" pages 760-764.	1-7



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"T" Inter document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

11 SEPTEMBER 1992

Date of mailing of the international search report

23 SEP 1992

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